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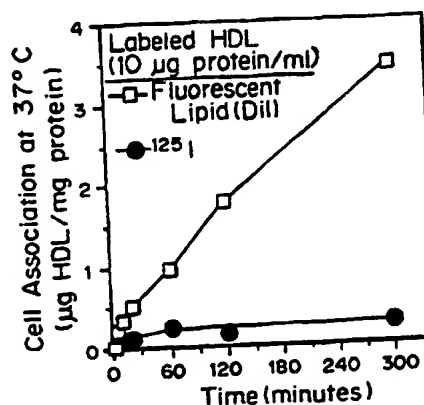
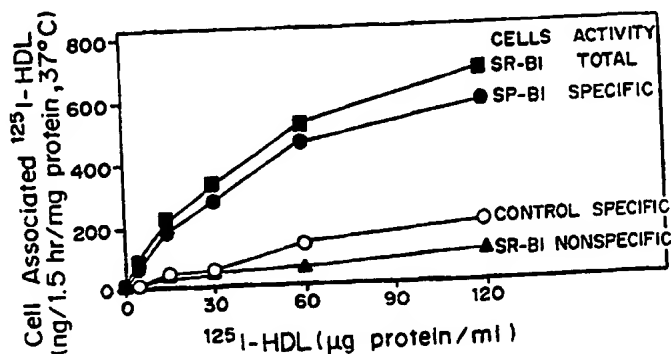
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(54) Title: **CLASS BI AND CI SCAVENGER RECEPTORS**

(57) Abstract

Two distinct scavenger receptor type proteins having high affinity for modified lipoproteins and other ligands have been isolated, characterized and cloned. HaSR-BI, an AcLDL and LDL binding scavenger receptor, which is distinct from the type I and type II macrophage scavenger receptors, has been isolated and characterized and DNA encoding the receptor cloned from a variant of Chinese Hamster Ovary Cells, designated Var-261. dSR-CI, a non-mammalian AcLDL binding scavenger receptor having high ligand affinity and broad specificity, was isolated from *Drosophila melanogaster*. The isolated receptors are useful in screening for drugs that inhibit uptake of cholesterol in endothelial or adipose cells or macrophages, respectively. They are also useful as probes for the isolation of other lipoprotein receptors and in research the roles of these receptors.



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## CLASS BI AND CI SCAVENGER RECEPTORS

## Background of the Invention

The present invention is generally in the area of new scavenger receptor proteins present on cells which can mediate lipid or lipoprotein uptake, genes encoding these proteins and methods of detection and use thereof.

The intercellular transport of lipids through the circulatory system requires the packaging of these hydrophobic molecules into water-soluble carriers, called lipoproteins, and the regulated targeting of these lipoproteins to appropriate tissues by receptor-mediated endocytic pathways. The most well characterized lipoprotein receptor is the LDL receptor, which binds to apolipoproteins B-100 (apoB-100) and E (apoE), which are constituents of low density lipoprotein, the principal cholesteryl-ester transporter in human plasma (LDL), very low-density lipoprotein, a triglyceride-rich carrier synthesized by the liver (VLDL), intermediate-density lipoprotein (IDL), and catabolized chylomicrons (dietary triglyceride-rich carriers synthesized by the liver).

All members of the LDL receptor gene family consist of the same basic structural motifs, shown in Figure 1a. Ligand-binding (complement-type) cysteine-rich repeats of approximately 40 amino acids are arranged in clusters (ligand-binding domains) that contain between two and eleven repeats. Ligand-binding domains are always followed by EGF-precursor homologous domains. In these domains, two EGF-like repeats are separated from a third EGF-repeat by a spacer region containing the YWTD motif. In LRP and gp330, EGF-precursor homologous domains are either followed by another ligand-binding domain or by a spacer region. The EGF-precursor homology domain, which precedes the plasma membrane, is separated from

the single membrane-spanning segment either by an O-linked sugar domain (in the LDL receptor and VLDL receptor) or by one (in *C. elegans* and gp330) or six EGF-repeats (in LRP). The cytoplasmic tails contain  
5 between one and three "NPXY" internalization signals required for clustering of the receptors in coated pits. In a later compartment of the secretory pathway, LRP is cleaved within the eighth EGF-precursor homology domain. The two subunits LRP-515  
10 and LRP-85 (indicated by the brackets) remain tightly and non-covalently associated. Only partial amino acid sequence of the vitellogenin receptor and of gp330 are available.

LDL receptors and most other mammalian cell-  
15 surface receptors that mediate binding and, in some cases, the endocytosis, adhesion, or signaling exhibit two common ligand-binding characteristics: high affinity and narrow specificity. However, two  
20 additional lipoprotein receptors have been identified which are characterized by high affinity and broad specificity: the macrophage scavenger receptors type I and type II.

Scavenger receptors mediate the endocytosis of chemically modified lipoproteins, such as acetylated  
25 LDL (AcLDL) and oxidized LDL (OxLDL), and have been implicated in the pathogenesis of atherosclerosis (Krieger and Herz, 1994 J. Annu. Rev. Biochem. 63, 601-637; Brown and Goldstein, 1983 Annu. Rev. Biochem. 52, 223-261; Steinberg et al., 1989 N. Engl. J. Med.  
30 320, 915-924). Macrophage scavenger receptors exhibit complex binding properties, including inhibition by a wide variety of polyanions, such as maleylated BSA (M-BSA) and certain polynucleotides and polysaccharides, as well as unusual ligand-cross competition (Freeman  
35 et al., 1991 Proc. Natl. Acad. Sci. U.S.A. 88, 4931-4935, Krieger and Herz, 1994). Several investigators have suggested that there may be at least three different classes of such receptors expressed on mammalian macrophages, shown in Figure 3, including

receptors which recognize either AcLDL or OxLDL, or both of these ligands (Sparrow et al., 1989 J. Biol. Chem. 264, 2599-2604; Arai et al., 1989 Biochem. Biophys. Res. Commun. 159, 1375-1382; Nagelkerke et al., 1983 J. Biol. Chem. 258, 12221-12227).

The first macrophage scavenger receptors to be purified and cloned were the mammalian type I and II receptors. These are trimeric integral membrane glycoproteins whose extracellular domains have been predicted to include  $\alpha$ -helical coiled-coil, collagenous and globular structures (Kodama et al., 1990 Nature 343, 531-535; Rohrer et al., 1990; Krieger and Herz, 1994). The collagenous domain, shared by the type I and type II receptors, apparently mediates the binding of polyanionic ligands (Acton et al., 1993 J. Biol. Chem. 268, 3530-3537; Doi et al., 1993 J. Biol. Chem. 268, 2126-2133). The type I and type II molecules, which are the products of alternative splicing of a single gene, are hereafter designated class A scavenger receptors (SR-AI and SR-AII). The class A receptors, which bind both AcLDL and OxLDL (Freeman et al., 1991), have been proposed to be involved in host defense and cell adhesion, as well as atherogenesis (Freeman et al., 1991; Krieger, 1992 Trends Biochem. Sci. 17, 141-146; Fraser et al., 1993 Nature 364, 343-346; Krieger and Herz, 1994).

Models of the predicted quaternary structures of the type I and type II macrophage scavenger receptors are shown in Figure 1B (AR-A, I, II & III). Both contain six domains, of which the first five are identical: the N-terminal cytoplasmic region, the transmembrane region, spacer,  $\alpha$ -helical coil, and collagen-like domains. The C-terminal sixth domain of the type I receptor is composed of an eight-residue spacer followed by a 102-amino acid cysteine-rich domain (SRCR), while the sixth domain of the type II receptor is only a short oligopeptide.

Using a murine macrophage cDNA library and a COS cell expression cloning technique, Endemann, Stanton

and colleagues, (Endemann, et al. 1993 J. Biol. Chem. 268, 11811-11816; Stanton, et al. J. Biol. Chem. 267, 22446-22451), reported the cloning of cDNAs encoding two additional proteins that can bind OxLDL. The binding of OxLDL to these proteins was not inhibited by AcLDL. These proteins are FcγRII-B2 (an Fc receptor) (Stanton et al., 1992) and CD36 (Endemann et al., 1993). The significance of the binding of OxLDL to FcγRII-B2 in transfected COS cells is unclear because FcγRII-B2 in macrophages apparently does not contribute significantly to OxLDL binding (Stanton et al., 1992). However, CD36 may play a quantitatively significant role in OxLDL binding by macrophages (Endemann et al., 1993). In addition to binding oxidized LDL, CD36 binds thrombospondin (Asch et al., 1987 J. Clin. Invest. 79, 1054-1061), collagen (Tandon et al., 1989 J. Biol. Chem. 264, 7576-7583), long-chain fatty acids (Abumrad et al., 1993 J. Biol. Chem. 268, 17665-17668) and *Plasmodium falciparum* infected erythrocytes (Oquendo et al., 1989 Cell 58, 95-101). CD36 is expressed in a variety of tissues, including adipose, and in macrophages, epithelial cells, monocytes, endothelial cells, platelets, and a wide variety of cultured lines (Abumrad et al., 1993; and see Greenwalt et al., 1992 Blood 80, 1105-1115 for review). Although the physiologic functions of CD36 are not known, it may serve as an adhesion molecule due to its collagen-binding properties. It is also been proposed to be a long-chain fatty acid transporter (Abumrad et al., 1993) and a signal transduction molecule (Ockenhouse et al., 1989 J. Clin. Invest. 84, 468-475; Huang et al., 1991), and may serve as a receptor on macrophages for senescent neutrophils (Savill et al., 1991 Chest 99, 7 (suppl)).

Modified lipoprotein scavenger receptor activity has also been observed in endothelial cells (Arai et al., 1989; Nagelkerke et al., 1983; Brown and Goldstein, 1983; Goldstein et al., 1979 Proc. Natl. Acad. Sci. U.S.A. 76, 333-337). The endothelial cell

activity apparently is not mediated by the class A scavenger receptors (Bickel et al., 1992 J. Clin. Invest. 90, 1450-1457; Arai et al., 1989; Nagelkerke et al., 1983; Via et al., 1992 The Faseb J. 6, A371), which are expressed almost exclusively by macrophages (Naito et al., 1991 Am. J. Pathol. 139, 1411-1423; Krieger and Herz, 1994). *In vivo* and *in vitro* studies suggest that there may be scavenger receptor genes expressed in endothelial cells and macrophages which differ from both the class A scavenger receptors and CD36 (Haberland et al., 1986 J. Clin. Inves. 77, 681-689; Via et al., 1992; Sparrow et al., 1989; Horiuchi et al., 1985 J. Biol. Chem. 259, 53-56; Arai et al., 1989; and see below). Via, Dressel and colleagues (Ottstad et al., 1992 Biochem J. 281, 745-751) and Schnitzer et al. 1992 J. Biol. Chem. 267, 24544-24553) have detected scavenger receptor-like binding by relatively small membrane associated proteins of 15-86 kD. In addition, the LDL receptor related protein (LRP) has been shown to bind lipoprotein remnant particles and a wide variety of other macromolecules. Both the mRNA encoding LRP and the LRP protein are found in many tissues and cell types (Herz, et al., 1988 EMBO J. 7:4119-4127; Moestrup, et al., 1992 Cell Tissue Res. 269:375-382), primarily the liver, the brain and the placenta. The predicted protein sequence of the LRP, shown in Figure 1A, consists of a series of distinctive domains or structural motifs, which are also found in the LDL receptor.

Based on the information known regarding the structures and functions of multiligand lipoprotein receptors present on macrophages, it would clearly be of benefit to isolate and clone other members of the lipoprotein receptor family present on macrophages, especially from non-mammalian species, in order to investigate which aspects of these molecules are most conserved and which portions can therefore be selectively targeted for stimulation or inhibition of

binding, and on other cell types, the structure and function of whose receptors are not characterized.

It is therefore an object of the present invention to provide the structure, amino acid  
5 sequence, and DNA sequence encoding a previously undescribed lipoprotein receptors present on mammalian cells.

It is another object of the present invention to provide the structure, amino acid sequence, and DNA  
10 sequence encoding a lipoprotein receptor present on insect macrophages.

It is a further object of the present invention to provide methods and reagents for use in isolating and characterizing lipoprotein receptors that are not  
15 type I and type II macrophage scavenger receptors nor classic LDL receptors.

It is yet a still further object of the present invention to provide methods and reagents for designing drugs that can stimulate or inhibit the  
20 binding of lipoprotein receptors that are not type I and type II macrophage scavenger receptors nor classic LDL receptors.

It is still another object of the present invention to provide a method and means for altering  
25 cholesterol uptake and transport by cells.

#### Summary of the Invention

Two distinct scavenger receptor type proteins having high affinity for modified lipoproteins and other ligands have been isolated, characterized and  
30 cloned. HaSR-BI, an AcLDL and LDL binding scavenger receptor, which is distinct from the type I and type II macrophage scavenger receptors, has been isolated and characterized and DNA encoding the receptor cloned from a variant of Chinese Hamster Ovary Cells,  
35 designated Var-261, and from murine cells. dSR-CI, a non-mammalian AcLDL binding scavenger receptor having



high ligand affinity and broad specificity, was isolated from *Drosophila melanogaster*.

It has been discovered that the SR-BI receptor is expressed principally in steroidogenic tissues and adrenal tissue and appears to mediate HDL-transfer and uptake of cholesterol. Competitive binding studies show that SR-BI binds LDL, modified LDL, negatively charged phospholipid, and HDL. Direct binding studies show that SR-BI binds HDL-lipid, without degradation of the HDL, and lipid is accumulated within cells expressing the receptor. These studies indicate that AR-BI plays a major role in transfer of cholesterol from the liver to the steroidogenic tissues, and that increased expression in the liver or other tissues may be useful in increasing uptake of cholesterol by cells expressing SR-BI, thereby decreasing levels in foam cells and deposition at sites involved in atherogenesis.

The presence of scavenger receptors on both mammalian and *Drosophila* macrophages indicates that they mediate critical, well-conserved functions, including pathogen recognition, and that they may have appeared early in the evolution of host defense systems. In this regard, it is known that postembryonic macrophage-like hemocytes in *Drosophila* participate in wound healing, encapsulation of pathogens, and phagocytosis. Due to the known association between atherosclerosis and macrophages, and the uptake of cholesterol by macrophages which is mediated by scavenger receptor proteins, the isolated receptors are useful in screening for drugs that inhibit uptake of cholesterol by cells expressing these receptors. Studies also demonstrate that a 600 bp *Bam*HI portion of the cDNA encoding SR-BI hybridizes under stringent conditions to a mRNA expressed in adipocytes. The hybridizing sequence does not express a protein immunoreactive with antibody to SR-BI. The protein encoded by the hybridizing sequence is referred to as SR-BII.

### Brief Description of the Drawings

Figure 1a is a schematic of the LDL receptors described in the background of the invention. Figure 1b is a schematic of the three classes of scavenger receptors, SR-A (I, II and III), SR-B (CD36), and SR-C.

Figures 2A, 2B, and 2C are graphs of the concentration dependence of  $^{125}\text{I}$ -AcLDL interaction with control cells, Var-261 and ldlA-7 (LDL receptor deficient control cells, also referred to as ldlA cells) cells, at 4°C and 37°C. Figure 2A is binding at 4°C; Figure 2B is binding plus uptake at 37°C; and Figure 2C is degradation at 37°C, measured as ng  $^{125}\text{I}$ -AcLDL/5 hr/mg cell protein versus  $^{125}\text{I}$ -AcLDL ( $\mu\text{g}$  protein/ml).

Figures 3A and 3B are graphs of the binding of  $^{125}\text{I}$ -AcLDL to haSR-BI expressed in transfected COS cells which do not otherwise express SR-BI (Figure 3A) and specificity of binding of  $^{125}\text{I}$ -AcLDL to Var-261 and ldlA [haSR-BI] cells (Figure 3B) measured as the percent of control binding in the presence of competitor: M-BSA (10  $\mu\text{g}/\text{ml}$ ), poly G (500  $\mu\text{g}/\text{ml}$ ), Fucoidin (200  $\mu\text{g}/\text{ml}$ ), Carrageenan (200  $\mu\text{g}/\text{ml}$ ), and LDL (500  $\mu\text{g}/\text{ml}$ ).

Figures 4A and 4B are graphs of the binding of  $^{125}\text{I}$ -AcLDL to CD36 expressed in transfected COS cells (Figure 4A) and specificity of binding of  $^{125}\text{I}$ -AcLDL to transfected COS cells (Figure 4B) measured as percent of control in the presence of a competitor: M-BSA (4  $\mu\text{g}/\text{ml}$ ), AcLDL (500  $\mu\text{g}/\text{ml}$ ), Fucoidin (200  $\mu\text{g}/\text{ml}$ ), Poly I (500  $\mu\text{g}/\text{ml}$ ), Poly G (500  $\mu\text{g}/\text{ml}$ ), and ReLPS (250  $\mu\text{g}/\text{ml}$ ).

Figure 5 is a graph of lipoprotein inhibition of  $^{125}\text{I}$ -AcLDL binding to haSR-BI and huCD36, measured as percent of control binding at 4°C, to either COS[haSR-BI] or COS[huCD36], alone, in the presence of AcLDL, OxLDL or LDL.

Figures 6A, 6B, and 6C are graphs of the concentration dependent binding, uptake and degradation of  $^{125}\text{I}$ -AcLDL to CHO[dSR-CI]-2.6a cells: Figure 6A is binding at 4°C; Figure 6B is binding plus uptake at 37°C; and Figure 6C is degradation at 37°C, measured as ng  $^{125}\text{I}$ -AcLDL/5 hr/mg cell protein versus  $^{125}\text{I}$ -AcLDL ( $\mu\text{g}$  protein/ml).

Figures 7a and 7b are graphs  $^{125}\text{I}$ -AcLDL binding, 4°C (% of control) as a function of liposome concentration ( $\mu\text{g}$  phospholipid/ml) (PS, circles; PI, triangles; PC, open circles), haSR-BI, Figure 7a; huCD36, Figure 7b.

Figure 7c is a graph of the effect of PS:PC ratio on inhibition of  $^{125}\text{I}$ -LDL binding to SR-BI,  $^{125}\text{I}$ -LDL binding at 4°C (% of control) to PS in liposomes (mole %).

Figure 7d is [ $^3\text{H}$ ]-liposome binding to haSR-BI, [ $^3\text{H}$ ]-liposome binding at 4°C ( $\mu\text{g}$  phospholipid/mg cell protein) versus [ $^3\text{H}$ ]-liposomes ( $\mu\text{g}$  phospholipid/ml) ([ $^3\text{H}$ ]PS, ldlA[haSR-BI] (circles); [ $^3\text{H}$ ]PS, ldlA (square); [ $^3\text{H}$ ]PC, ldlA[haSR-BI] (open circles)).

Figure 7e is a graph of the specificity of binding to SR-BI, binding of haSR-BI at 4°C (% of control) versus competitor (150  $\mu\text{g}/\text{ml}$ ), [ $^3\text{H}$ ]PS (light bar) and  $^{125}\text{I}$ -LDL (dark bar).

Figure 8a is a graph of the cell associated  $^{125}\text{I}$ -HDL (ng/1.5 hr/mg protein, 37°C) as a function of  $^{125}\text{I}$ -HDL ( $\mu\text{g}$  protein/ml), for SR-BI transfected CHO cells and untransfected controls (ldlA-7).

Figure 8b is a graph of SR-BI mediated delivery of fluorescent lipid from HDL to cells expressing SR-BI on their surface, cell association at 37°C ( $\mu\text{g}$  HDL/mg protein) as a function of time (minutes), for fluorescent lipid-labeled HDL (squares) and  $^{125}\text{I}$ -labeled HDL (circles).

Figure 9 is a schematic of potential roles of SR-BI in HDL metabolism.

### Detailed Description of the Invention

In order to isolate, characterize, and clone the genes for new scavenger receptors and other lipoprotein receptors, cDNA and genomic libraries are prepared from cells in which activities have been identified which are characteristic of scavenger receptors: binding to lipoproteins such as LDL, HDL, AcLDL and/or oxidized-LDL; hybridization screening of the genomic libraries using probes generated from the nucleic acid sequences of cloned receptors; expression cloning by transient expression in COS cells and/or expression cloning by stable expression in CHO cells; analysis of the cloned cDNA: verification, sequencing and sequence analysis; identification and isolation of the genomic DNA including regulatory sequences; immunochemical analysis of the structure and biosynthesis of the new scavenger receptors; and characterization of the binding properties of the new receptors and comparisons with type I and type II receptors.

#### **I. Isolation and Characterization of a mammalian scavenger receptor protein on CHO cells.**

To extend the analysis of the structure and function of mammalian modified lipoprotein scavenger receptors, a variant Chinese hamster ovary cell line, Var-261, which, based on ligand specificity, expresses an apparently novel polyanion binding scavenger receptor, was identified and characterized. The cDNA for a scavenger receptor, haSR-BI, which is a new member of the CD36 family of membrane proteins (class B scavenger receptors), was isolated from the Var-261 cells. Although isolated from the same cells, haSR-BI is not responsible for the novel polyanion binding receptor activity of Var-261 cells, and is present in normal cells present in a variety of tissues, as discussed below.

The ligand specificities of CD36 and haSR-BI expressed in transfected cell lines was compared with that of Var-261 cells. haSR-BI differs from CD36 and

other modified lipoprotein receptors described to date in that its binding of AcLDL is inhibited by native LDL. SR-BI also binds HDL and mediates uptake of lipid from HDL into the cell.

5       The cDNA encoding SR-BI yields a predicted protein sequence of 509 amino acids which is approximately 30% identical to those of the three previously identified CD36 family members. Northern blot analysis of murine tissues shows that SR-BI is  
10   most abundantly expressed in fat and is present at moderate levels in lung and liver. Furthermore, SR-BI mRNA expression is induced upon differentiation of 3T3-L1 cells into adipocytes. Both SR-BI and CD36 display high affinity binding for acetylated LDL with  
15   an apparent dissociation constant in the range of approximately 5  $\mu$ g protein/ml. The ligand binding specificities of CD36 and SR-BI, determined by competition assays, are similar, but not identical: both bind modified proteins (acetylated LDL,  
20   maleylated BSA), but not the broad array of other polyanions (e.g. fucoidin, polyinosinic acid, polyguanosinic acid) which are ligands of the class A receptors. SR-BI displays high affinity and saturable binding of HDL which is not accompanied by cellular  
25   degradation of the HDL. HDL inhibits binding of AcLDL to CD36, suggesting that it binds HDL, similarly to SR-BI. Native LDL, which does not compete for the binding of acetylated LDL to either class A receptors, CD36 or Var-261 cells, unexpectedly competes for  
30   binding to SR-BI. SR-BI and CD36 therefore define a second class of scavenger receptors, designated class B, which are referred to as members of the CD36 family which can bind to modified LDL. The ability of other known members of the CD36 family to bind to modified  
35   LDLs has not been reported. Class B scavenger receptors may play a role in the *in vivo* and *in vitro* uptake of modified proteins previously described by Haberland et al., 1989 J. Immunol. 142, 855-862; Villaschi et al., 1986 Microvasc. Res. 32, 190-199;

Horiuchi et al., 1985; Predescu et al., 1988 J. Cell Biol. 107, 1729-1738).

These methods and conclusions are described in greater detail below. Abbreviations: LDL (low density lipoprotein), OxLDL (oxidized LDL), AcLDL (acetylated LDL), M-BSA (maleylated BSA), CHO (Chinese hamster ovary), haSR-BI (hamster scavenger receptor type BI), mSR-AII (murine scavenger receptor type AII), huCD36 (human CD36).

## 10       **Materials and Methods**

### Materials

LDL, AcLDL, <sup>125</sup>I-labeled AcLDL (100-400 cpm/ng protein), and newborn calf and human lipoprotein-deficient sera were prepared as described by Goldstein et al., 1983 Methods Enzymol. 98, 241-260; Krieger, 1983 Cell 33, 413-422. For some preparations of LDL and AcLDL, the following additional precautions were taken to prevent inadvertent oxidation: 1) 20  $\mu$ M  $\beta$ -hydroxytoluene was added to the plasma before separation of the lipoproteins on the gradient, 2) one hour prior to all dialysis steps the dialysis solutions were degassed by vacuum, followed by purging with nitrogen gas 3) whenever possible purification procedures were performed in the dark or low light, and 4) the preparations were stored in the dark under argon. OxLDL was prepared by dialyzing 1 ml of LDL (4-10 mg/ml) (prepared without  $\beta$ -hydroxytoluene) against saline solution containing 5  $\mu$ M Cu<sub>2</sub>SO<sub>4</sub> (2 x 500 ml) for 24 to 48 hours at 4°C. Using a lipid peroxidation assay (El-Saadani et al., 1989), very little inadvertent oxidation in LDL and AcLDL preparations isolated with or without the precautions listed above was measured. Polyriboguanilyc acid (poly G), polyriboinosinic acid (poly I), fucoidin, dextran sulfate, chondroitin sulfate, ReLPS (*S. minnesota* Re-595) and carrageenan (type III Kappa) were obtained from Sigma Chemical Co. Maleylated BSA (M-BSA) was prepared as described by Goldstein et al., 1979. The CD36 expression vector was a gift from B. Seed

(Massachusetts General Hospital, Boston). 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) and 3-pyrenemethyl-23,24-dinor-5-cholesten-22-oate-3 $\beta$ -yl oleate (PMCAO) were obtained from Molecular Probes (Eugene, OR) and were used to prepare fluorescently labeled AcLDL as described by Krieger et al., 1979 J. Supra. Struct. 10, 467-478; Krieger, 1986 Meth. Enzymol. 128, 608-613; Pitas et al., 1985 J. Cell. Biol. 100, 103-117; Kingsley and Krieger, 1984 Proc Natl. Acad. Sci. USA 88, 7844-7848. Compactin and dioctadecylamidoglycylspermine (DOGS) were generous gifts from A. Endo and J. R. Falck, respectively.

#### Cell culture and transfections.

CHO, 1d1A (clone 7) and COS M6 cells were grown in culture as described by Krieger et al., 1983 Proc. Natl. Acad. Sci. USA 80, 5607-5611; Acton et al., 1993. All incubations with cells were performed at 37°C in a humidified 5% CO<sub>2</sub>/95% air incubator unless otherwise noted. COS M6 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2 mM glutamine (medium A). For transient transfections other than in the expression cloning experiments, 1 x 10<sup>6</sup> COS cells were plated in 100 mm dishes in medium A on day 0. On day 1, plasmid DNA (between 2 and 10  $\mu$ g in 1.9 ml phosphate buffered saline [PBS, calcium/magnesium free]) was mixed with 100  $\mu$ l of 10 mg/ml DEAE-dextran (Pharmacia) and then added to each dish, which was then incubated at 37°C with gentle shaking every 10 minutes. After 30 minutes, 8 ml of medium A supplemented with 80  $\mu$ M chloroquine (Sigma) were added and the cells were incubated for another 2.5 hrs. The medium was removed and the cells were shocked with 5 ml of 10% (v/v) dimethylsulfoxide in medium A for 2.5 minutes. This medium was quickly removed by aspiration, the cells were washed with 10 ml of PBS (containing calcium and magnesium), and 10 ml of fresh

medium A were added. On day 2 the cells were harvested with trypsin and replated ( $0.5 - 1 \times 10^6$  cells/well in 6-well dishes) in medium A containing 1 mM sodium butyrate. On day 3, the cells were assayed for ligand binding at 4°C and for binding plus uptake (binding/uptake) and degradation at 37°C. Stable transfections were performed as follows: ldlA cells were plated at  $1 \times 10^6$  cells per 100 mm dish in medium B (Ham's F12 containing 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine) supplemented with 10% fetal calf serum. On day 2, the cells were washed three times with medium B. A complex of DNA and DOGS (dioctadecylamidoglycylspermine, which was a gift from J.R. Falck) was prepared by adding 150 µl of a DNA solution (12 µg of plasmid and 1.2 µg of supercoiled pSV2neo in 300 mM NaCl ) to 150 µl of solution A (0.33 mg/ml DOGS, 300 mM NaCl). The DNA/DOGS complex was added to the cells in 3.6 ml of serum-free Opti-MEM medium (GIBCO) containing penicillin (100 U/ml) and streptomycin (100 µg/ml). After a 14 hr transfection period, the cells were washed three times with medium B and incubated in medium B plus 10% fetal calf serum for one day before being replated at a density of  $3 \times 10^6$  cells per 100 mm dish. One day later, the culture medium was replaced with selection medium, medium B supplemented with 10% fetal calf serum and 0.25 mg/ml G418. This medium was changed every two to three days, and G418-resistant colonies appeared 10 to 12 days after transfection. The colonies were screened for DiI-AcLDL endocytosis and positive colonies were picked and grown to mass culture for further analysis.

#### Isolation of Var-261 cells.

Var-261 cells, a rare spontaneously arising variant expressing a novel scavenger receptor activity, were isolated during a transfection/selection experiment as follows. Human liver genomic DNA prepared as described by Sege et al., 1984 Nature 307, 742-745, was transfected into



ldlA (clone 7) cells, a CHO cell mutant clone whose defective LDL receptor gene results in an essentially LDL receptor-negative phenotype (Sege, et al., 1986 Mol. Cell. Biol. 6, 3268-3277; Kingsley et al., 1986 Mol. Cell. Biol. 6, 2734-2737; Kingsley and Krieger, 1984). After transfection, variants expressing scavenger receptor activity were isolated by growing the cells in MAC selection medium (Penman et al., 1991 J. Biol. Chem. 266, 23985-23993) [250  $\mu$ M Mevalonate, 3  $\mu$ g protein/ml of AcLDL, 40  $\mu$ M Compactin, and 3% (v/v) newborn calf lipoprotein-deficient serum in medium B. In MAC medium, endogenous cholesterol synthesis is inhibited by compactin and only those cells that are able to obtain cholesterol by the endocytosis of AcLDL can survive. The parental ldlA cells used for the transfection cannot grow in MAC medium. After incubation in MAC medium for 29 days, surviving colonies were re-fed with medium B supplemented with 3% (v/v) newborn calf lipoprotein-deficient serum containing AcLDL which was fluorescently labeled by reconstitution of the lipid core (Krieger, 1986) with the pyrene-based lipophilic dye PMCAO (Krieger et al., 1979). Colonies which accumulated significant amounts of fluorescence from the lipoprotein, as determined by fluorescence microscopy (Krieger et al., 1981 J. Mol. Biol. 150, 167-184), were harvested and maintained in MAC selection medium. One colony, designated Var-261, exhibited significant levels of AcLDL binding, uptake and degradation activity which were dramatically greater than those in either the parental ldlA cells or any of the other colonies. Southern blot analysis using human genomic DNA as a probe showed that there was no detectable human-specific repeat DNA in Var-261 cells. Thus, Var-261 cells are presumably rare, spontaneously arising variants which express an endogenous hamster scavenger receptor gene. This expression could be due to the activation of an otherwise silent gene. Alternatively, it might be due to a spontaneous mutation which conferred novel

activity on an endogenous protein with some other function (e.g., see Faust and Krieger, 1987 J. Biol. Chem. 262, 1996-2004, and Chen et al., 1990 J. Biol. Chem. 265, 3116-3123).

5           <sup>125</sup>I-AcLDL Binding, Uptake and Degradation Assays.

Scavenger receptor activities at 37°C were measured by ligand binding, uptake and degradation assays as described by Krieger, 1983; Freeman et al., 1991). Although binding and uptake at 37°C were  
10 determined separately using dextran-sulfate to free surface bound lipoproteins from the cells as described for LDL receptor assays by Goldstein et al., 1983; Basu et al., 1983 Science 219, 871-873), this method for separating bound from internalized ligand for <sup>125</sup>I-AcLDL and scavenger receptors has not been  
15 independently validated. Accordingly, the values for binding and uptake were combined and are presented as binding plus uptake observed after a 5 hour incubation and are expressed as ng of <sup>125</sup>I-AcLDL protein per 5 hr  
20 per mg cell protein. Degradation activity is expressed as ng of <sup>125</sup>I-AcLDL protein degraded in 5 hours per mg of cell protein. The specific, high affinity values presented represent the differences between the results obtained in the presence (single  
25 determinations) and absence (duplicate determinations) of excess unlabeled competing ligand (75 to 200 µg/ml of M-BSA). Cell surface 4°C binding was assayed using either method A or method B as indicated. In method A, cells were prechilled on ice for 15 min, re-fed  
30 with <sup>125</sup>I-AcLDL in ice-cold medium B supplemented with 10% (v/v) fetal bovine serum, with or without 75 - 200 µg/ml unlabeled M-BSA, and incubated 2 hr at 4°C on a shaker. Cells were then washed rapidly three times with Tris wash buffer (50 mM Tris-HCl, 0.15 M NaCl, pH  
35 7.4) containing 2 mg/ml BSA, followed by two 5 min washes, and two rapid washes with Tris wash buffer without BSA. The cells were solubilized in 1 ml of 0.1 N NaOH for 20 min at room temperature on a shaker, 30 µl were removed for protein determination, and the

radioactivity in the remainder was determined using a LKB gamma counter. Method B differed from method A in that the cells were prechilled for 45 minutes, the medium contained 10 mM HEPES and 5% (v/v) human lipoprotein-deficient serum rather than fetal bovine serum, and the cell-associated radioactivity released by treatment with dextran sulfate was measured as described by Krieger, 1983; Freeman et al., 1991).

Preparation of Var-261 cDNA library.

10 Poly A<sup>+</sup> mRNA was isolated from Var-261 cells using standard procedures (Sambrook, Fritsch, and Maniatis. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press (1989)). This mRNA was used to synthesize double-  
15 stranded cDNA using a Not I unidirectional primer (Invitrogen) according to the method of Aruffo and Seed, 1987 Immunology 84, 8573-8577. BstXI linkers (Invitrogen) were added to the cDNA. The isolated cDNA was digested with Not I, size selected from  
20 agarose gels (in three size groups: 1 to 2 kb, 2 to 4 kb, greater than 4 kb), and ligated into the expression vector pcDNA I (Invitrogen). The average insert size for the entire Var-261 unidirectional library was approximately 1.5 kb. The DNAs for  
25 transfection experiments were prepared as follows. *E. coli* strain MC1061/P3 was transformed with the cDNA expression plasmids by electroporation and the transformed cells were plated on LB-A/T plates (Luria broth with ampicillin (15 µg/ml)/tetracycline (8  
30 µg/ml)) (Sigma) to obtain isolated colonies at densities of approximately 5,000 per 150 mm dish. Each plate, representing one pool, was scraped to recover the bacteria and a pool of library plasmids was isolated from each bacterial mixture using a  
35 midiprep method (miniprep method scaled up 5 to 10 fold (Sambrook et al., 1989)).

Expression cloning by transient expression in COS cells.

On day 0, COS M6 cells were plated in 35 mm  
40 culture dishes (3 to 4 x 10<sup>5</sup> cells/dish) in medium A.

On day 1, the cells in each dish were transfected with 0.5  $\mu$ g/dish of expression library DNA from a single pool following the DEAE-dextran method of Cullen 1987 Methods in Enz. 152, 684-704. On day 2, monolayers  
5 were re-fed with medium A containing 1 mM sodium butyrate (modified medium A). On day 3, the monolayers were re-fed with modified medium A containing one to five  $\mu$ g protein/ml of DiI-labeled AcLDL (DiI-AcLDL). After a 5 hr incubation at 37°C,  
10 the plates were washed two times with PBS and the cells were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. The presence of fluorescent DiI in the fixed cells was determined by visual inspection using a Leitz inverted fluorescence  
15 microscope with a rhodamine filter package as described by Kingsley and Krieger, (1984). Each transfection experiment included negative control transfections with empty vector pcDNA I (0 to 20 DiI-positive cells per dish) and positive control  
20 transfections with a mixture of a pcDNA I-based bovine type II macrophage scavenger receptor expression vector, pXSR3 (Rohrer et al., 1990) and the empty vector pcDNA I (1:5000 ratio, 200 to 400 DiI-positive cells per dish). A positive pool was serially  
25 subdivided and retested to permit the purification of the single positive plasmid, phaSR-BI.

#### Northern blot analysis.

0.5 micrograms of poly(A)+ RNA prepared from different murine tissues or from 3T3-L1 cells on zero,  
30 two, four, six or eight days after initiation of differentiation into adipocytes as described by Baldini et al., 1992 Proc. Natl. Acad. Sci. U.S.A. 89, 5049-5052, was fractionated on a formaldehyde/agarose gel (1.0%) and then blotted and fixed onto a  
35 Biotrans™ nylon membrane. The blots were hybridized with the indicated probes that were <sup>32</sup>P-labeled (2 x 10<sup>6</sup> dpm/ml, random-primed labeling system). The hybridization and washing conditions, at 42°C and 50°C, respectively, were performed as described by

Charron et al., 1989 Proc. Natl. Acad. Sci. U.S.A. 86, 2535-2539. The probe for haSR-BI mRNA analysis was a 0.6 kb BamHI fragment from the cDNA's coding region. The coding region of murine cytosolic hsp70 gene (Hunt and Calderwood, 1990 Gene 87, 199-204) was used as a control probe for equal mRNA loading.

SR-BI protein in tissues was detected by blotting with polyclonal antibodies to SR-BI.

#### HDL Binding Studies

HDL and VLDL binding to SR-BI and CD36 were conducted as described for LDL and modified LDL.

Studies conducted to determine if the HDL which is bound to SR-BI is degraded or recycled and if lipid which is bound to the HDL is transferred into the cells were conducted using fluorescent lipid-labeled HDL and  $^{125}\text{I}$ -HDL added to cultures of transfected or untransfected ldlA-7 at a single concentration (10  $\mu\text{g}$  protein/ml). HDL associated with the cells was measured over time. A steady state was reached in approximately thirty minutes to one hour. A fluorescent ligand, DiI, described above with reference to LDL, was used as a marker for lipid (for example, cholesterol or cholesterol ester) uptake by the cell. Increasing concentration of DiI indicates that lipid is being transferred from the HDL to the receptor, then being internalized by the cell. The DiI-depleted HDL is then released and replaced by another HDL molecule bound to lipid.

#### Phospholipid Binding and Competition Assays

Materials- Reagents (and sources) were acetic subhydride (Mallinckrodt, Inc., Paris, KY); egg phosphatidylcholine, egg phosphatidic acid, liver phosphatidylinositol, brain phosphatidylserine, egg phosphatidylethanolamine, and brain sphingomyelin (Avanti Polar Lipids, Inc., Alabaster, AL); polycarbonate membrane filters (Poretica Corp., Livermore, CA); sodium [ $^{125}\text{I}$ ]iodide and 1,2-dipalmitoyl-L-3-phosphatidyl [*N*-methyl- $^3\text{H}$ ] choline ([ $^3\text{H}$ ]DPPC) (Amersham Corp.); DEAE-dextran (Pharmacia

Biotech. Inc., Ham's F-12 medium, Dulbecco's modified Eagle's medium, fetal bovine serum, and trypsin/EDTA ((JRII Bio-science, Lenera, KS); and penicillin/streptomycin, glutamine, and GIBCO BRL G-418 sulfate (Life Technologies, Inc.). All other reagents and supplies were purchased from Sigma or were obtained as described previously (Krieger, 1983). Human LDL, AcLDL, <sup>125</sup>I-labeled LDL, and <sup>125</sup>I-labeled AcLDL (90-300 cpm/ng protein) were prepared essentially as described previously (Goldstein et al., 1983; Krieger, 1983; Acton et al., 1994).

*Phospholipid Liposome Preparation.*

Unilamellar liposomes were made by extrusion through polycarbonate membranes (Szoka et al., 1980). Phospholipid liposomes were prepared containing the indicated phospholipid, phosphatidycholine, and free cholesterol in a molar ratio of 1:1:1. The lipids were mixed in chloroform and dried by rotary evaporation for 30 min. For preparation of radiolabeled liposomes, 50-75  $\mu$ Ci of [<sup>3</sup>H]DPPC(62 Ci/mmol) were added to the lipid mixtures before drying. The dried lipids were resuspended in 150 mM NaCl, 0.1 mM EDTA, 10 mM HEPES, pH 7.5 (Buffer A). Once the samples were fully hydrated, they were extruded through 0.1- $\mu$  pore size polycarbonate membranes using a mini-extruder device (Avanti Polar Lipids, Inc., Alabaster, AL). After extrusion, liposomes were dialyzed against Buffer A and then stored under nitrogen at 4°C until use. Liposomes were used within 2 weeks of preparation. The final phospholipid concentration was determined by the method of Bartlett (1959). The average diameters of unlabeled liposomes, which were determined from either two or three independent preparations using light scattering with a Coulter N4 plus light scatterer apparatus (Coulter Electronics Inc., Hialeah, FL), were: PS, 105; PC, 114; PA, 125; PE, 129; PI, 113; and SM, 131 nm. The number of phospholipid molecules/PS liposome was calculated as follows. Cross-sectional

areas for cholesterol and phospholipid molecules in hydrated bilayers are assumed to be  $0.35 \text{ nm}^2$  and  $0.47 \text{ nm}^2$ , respectively (Levine and Wilklus, 1971); assuming an homogenous distribution of the components

5 throughout the PS/PC/cholesterol (1:1:1) liposomes, 73% of the surface area ( $4\pi r^2 \times 2$  (bilayer)  $\times 0.73 = 50477 \text{ nm}^2$ ) was phospholipid, or 107,398 phospholipid molecules/liposome ( $50477 \text{ nm}^2 / 0.47 \text{ nm}^2$ ). Based on an average phospholipid mass of 785 g/mol, a liposome  
10 concentration of  $10 \text{ } \mu\text{g}$  phospholipid/ml converts to 0.12 nM in liposome particles.

*Ligand Binding Assays.* On Day 0, ldlA and ldlA[haSR-BI] cells were plated ( $2.5 \times 10^6$  cells/well in six-well dishes) in medium A or B, respectively,  
15 and the assay was performed on day 2. Transfected COS cells were prepared as described above. Binding assays were performed as described above, with the following minor modifications. Cells were prechilled on ice for 30 min., incubated with the indicated  
20 radiolabeled ligands ( $^{125}\text{I}$ -LDL,  $^{125}\text{I}$ -AcLDL, or  $^3\text{H}$ -labeled liposomes) in ice-cold medium D (Ham's F-12 containing 0.5% (w/v) fatty acid free bovine serum albumin (FAF-BSA) and 10 mM HEPES, pH 7.4), with or without unlabeled competitors, for 2 h at  $4^\circ\text{C}$  with  
25 gentle shaking. Cells were then washed twice with Tris wash buffer (60 mM Tris-HCl, 0.15 M NaCl, pH 7.4) containing 2 mg/ml FAF-BSA, followed by one rapid wash with Tris wash buffer without FAF-BSA. The cells were then solubilized with 0.1 N NaCl, and radioactivity  
30 and protein determinations were made as described above. The specific, high affinity ligand binding activities shown represent the differences between values obtained in the absence (total binding) and presence (nonspecific binding) of an excess of the  
35 indicated unlabeled ligands. Nonspecific binding of [ $^3\text{H}$ ]PS liposomes to cells were generally low. The binding values are expressed as nanograms of bound  $^{125}\text{I}$ -labeled protein or ng of total phospholipids from  $^3\text{H}$ -labeled liposomes/milligram of cell protein.

Other procedures.

Protein concentrations were determined by the method of Lowry et al. 1951 J. Biol. Chem. 193, 265-275. DNA sequencing was performed using Sequenase 2.0 kit according to manufacturers instructions and the reported results for the coding region were verified by determining the sequence from both strands of the template. Oligonucleotide primers were prepared in the MIT Biopolymers Laboratory. Polymerase chain reaction (PCR) was used to generate a fragment of the hamster class A scavenger receptor DNA as follows. Primers based on the sequence of one exon of the murine scavenger receptor gene (Ashkenas et al., 1993 J. Lipid Res. 34, 983-1000) (5' AATGAAGAACTGCTTAGTTT 3' (Sequence ID No. 1) and 5' AATCAAGGAATTTAACTG 3' (Sequence ID No. 2)) were used to amplify a fragment of the expected size (240 base pairs) from hamster *ldlA* cell genomic DNA. This amplicon was cloned into pCR1000 (Invitrogen) to generate the plasmid pSA1, which was sequenced.

**Results**Isolation and Characterization of variant Var-261*High affinity binding, uptake and degradation of AcLDL*

The LDL receptor-deficient CHO cell line *ldlA* was processed and subjected to nutritional selection for rare variants which expressed endocytic receptors for AcLDL. One of the isolates, designated Var-261, was examined further. Var-261 and *ldlA* cells were plated on day 0 at 350,000 and 250,000 cells/well, respectively, in 6-well dishes in medium B plus 3% (v/v) newborn calf lipoprotein-deficient serum, and assayed on day 2 as described above. <sup>125</sup>I-AcLDL binding at 4°C for 2 hrs was measured using method B. <sup>125</sup>I-AcLDL binding plus uptake and degradation at 37°C was measured after a 5 hr incubation. The high affinity values shown represent the differences between measurements made in the absence (duplicate incubations) and presence (single incubations) of an excess of the unlabeled competitor M-BSA (100 µg/ml).



As shown by Figure 2A, these cells exhibited high affinity, saturable  $^{125}\text{I}$ -AcLDL binding at 4°C. The binding at 37°C and subsequent uptake shown in Figure 2B and lysosomal degradation shown in Figure 2C were characteristic of receptor-mediated endocytosis. Degradation, but not binding plus uptake, at 37°C was inhibited by the lysosomotropic drug chloroquine. In contrast, there was virtually no high affinity binding, uptake, or degradation of  $^{125}\text{I}$ -AcLDL by the parental ldlA cells or wild-type CHO cells. The scavenger receptor activity in Var-261 cells was not suppressed by the addition of sterols to the medium.

*Broad polyanion binding specificity*

A hallmark of macrophage scavenger receptors is their broad polyanion binding specificity, usually assessed by competition with polyanionic ligands for  $^{125}\text{I}$ -AcLDL binding, uptake and degradation. For further characterization, the ligand specificity of the scavenger receptor activity of Var-261 cells was compared with that of transfected COS cells transiently expressing the murine type II macrophage scavenger receptor, a class A scavenger receptor (mSR-AII).

COS cells were transfected on day 1 with murine scavenger receptor type II. On day 2 the transfected COS cells and Var-261 cells were set in 6-well dishes ( $1 \times 10^6$  and  $0.5 \times 10^6$  cells/well, respectively). On day 3,  $^{125}\text{I}$ -AcLDL (5  $\mu\text{g/ml}$ ) in medium B plus 10% fetal bovine serum was added to the cells and degradation was measured after a 5 hr incubation at 37°C in the absence (duplicate determinations) or presence (duplicate determinations) of the indicated amounts of polyanions. The 100% of control values (ng/5 hr/mg cell protein) from the two experiments were: Var-261, 606 and 473; and COS[mSR-AII], 1982 and 976. For the Var-261 and COS[mSR-AII] cells, the 100% of control values were: 321 and 390; and 848 and 1014, respectively.

The classic scavenger receptor polyanionic ligands AcLDL, fucoidin, and poly G were all effective competitors of  $^{125}\text{I}$ -AcLDL degradation by both Var-261 and the transfected COS cells, and native LDL did not inhibit the activity in either cell type. Nevertheless, the binding specificity in Var-261 cells differed from that in macrophages and for class A receptors in several critical ways. Poly I, a very effective competitor of class A scavenger receptors (greater than 50% inhibition at 2.5  $\mu\text{g/ml}$ ) only partially inhibited the activity in Var-261 cells (34% inhibition at 400  $\mu\text{g/ml}$ ). In contrast, maleylated BSA (M-BSA) inhibited the scavenger receptor activity in Var-261 cells at concentrations far lower than those required for inhibition of the murine type II receptors. The ReLPS form of endotoxin, which is a scavenger receptor competitor exhibiting complex binding properties for class A scavenger receptors (Ashkenas et al., 1993), only partly inhibited the activity in Var-261 cells at the high concentration of 250  $\mu\text{g/ml}$ .

*Determination if hamster scavenger receptor DNA is expressed in Var-261 cells*

The striking differences in ligand specificities between the scavenger receptor activity in Var-261 cells and those exhibited by the murine and bovine class A scavenger receptors (Ashkenas et al., 1993; Kodama et al., 1990; Rohrer et al., 1990) strongly suggested that the Var-261 cells did not express class A receptors. However, there was a possibility that the novel specificity was merely a consequence of species differences (e.g., hamster-derived Var-261 cells vs. murine SR-AII). To determine if this was the case, a 240 base pair fragment of the hamster class A macrophage scavenger receptor gene was isolated by polymerase chain reaction (PCR) using genomic CHO cell DNA as a template. This hamster gene fragment, which is found in both type I and II macrophage scavenger receptors, was cloned and sequenced. This fragment has between 75%-85%

nucleotide sequence identity with its human, murine, rabbit and bovine counterparts (Kodama et al., 1990; Rohrer et al., 1990; Ashkenas et al., 1993; Matsumoto et al., 1990 Proc. Natl. Acad. Sci. USA 87, 9133-9137; 5 Bickel and Freeman, 1992) and, with the exception of the amino acid corresponding to murine position 117, was consistent with the previously identified consensus sequence defined for residues 95 - 159 (Ashkenas et al., 1993).

10 This hamster fragment was used as a probe in Northern analysis of Var-261 cell mRNA. Under conditions where the hamster probe recognized murine scavenger receptor mRNA from CHO cells transfected with the murine scavenger receptor cDNA (Ashkenas et 15 al., 1993), no signal was detected in the hamster-derived Var-261 or control CHO cell mRNA, even after extensive overexposure of the film. Thus, Var-261 cells do not express significant amounts of the hamster type I or II class A receptors and must 20 instead express a different scavenger receptor.

*Cloning of haSR-BI receptor cDNA from Var-261*

In an attempt to clone a cDNA encoding this apparently new scavenger receptor, a cDNA expression 25 library was prepared from Var-261 poly A<sup>+</sup> mRNA, the library was divided into small pools (approximately 5000 clones/pool), the pools were transfected into COS cells and the transiently transfected cells visually screened for endocytosis of fluorescent AcLDL (DiI-AcLDL). A receptor-positive pool was obtained after 30 screening approximately 450,000 clones and this pool was subdivided repeatedly until a single functional plasmid (designated phaSR-BI for plasmid encoding hamster scavenger receptor type BI) was obtained.

35 Figures 3A and 3B are graphs of <sup>125</sup>I-AcLDL binding to haSR-BI expressed in transfected COS cells: Figure 3A demonstrates the concentration dependence in COS[haSR-BI] cells and Figure 3B demonstrates the ligand specificity in ldlA[haSR-BI] cells. To measure

binding of AcLDL, on day 1, COS cells were transfected with the expression vector for the hamster scavenger receptor type BI (haSR-BI). On day 2 the transfected COS cells were plated in 6-well dishes ( $1 \times 10^6$  cells/well in medium A plus 1 mM sodium butyrate). On day 3 between 0 and 50  $\mu\text{g/ml}$  of  $^{125}\text{I}$ -AcLDL were added to the monolayers at  $4^\circ\text{C}$  and binding was measured using Method A. The high affinity values shown in the Figures represent the differences between measurements made in the absence (duplicate incubations) and presence (single incubations) of an excess of the unlabeled competitor M-BSA (200  $\mu\text{g/ml}$ ). To measure binding specificity, on day 1, Var-261 and ldlA[haSR-BI] cells were plated in 6-well dishes ( $0.2 \times 10^6$  cells/well in MAC medium or medium B containing 10% fetal bovine serum and 0.25 mg/ml G418, respectively). On day 3 the binding of  $^{125}\text{I}$ -AcLDL (5  $\mu\text{g/ml}$ ) at  $4^\circ\text{C}$  in the absence or presence of the indicated competitors was measured using Method A. The values shown in the Figures represent the averages of duplicate determinations. The 100% of control values for Var-261 and ldlA[haSR-BI] were 1809 and 293 ng/mg cell protein, respectively.

The HaSR-BI plasmid conferred high affinity ( $K_d(4^\circ\text{C})$  approximately 5  $\mu\text{g}$  protein/ml)  $^{125}\text{I}$ -AcLDL binding on transiently transfected COS cells, as shown by Figure 3A. The ligand specificity of haSR-BI expressed in stably transfected ldlA cells was compared to that of the Var-261 cells, as shown in Figure 3B. M-BSA inhibited the binding of  $^{125}\text{I}$ -AcLDL to cells expressing haSR-BI and to Var-261 cells. Unexpectedly, haSR-BI was not inhibited by poly G, fucoidin or carrageenan (another classic scavenger receptor ligand (Brown and Goldstein, 1983)), which were competitors for the activity in Var-261 cells. In addition, LDL did not inhibit the binding of  $^{125}\text{I}$ -AcLDL to Var-261 cells, but was a competitor for cells expressing haSR-BI. There is no other case in which native LDL has been reported to block scavenger

receptor activity. Thus, the specificity of the haSR-BI differs dramatically from that reported for any previously described scavenger receptor, including that in Var-261 cells. Since the ligand specificities of the class B receptors described in more detail below are very different from the activity in Var-261 cells, it seems unlikely that either of these two class B scavenger receptors accounts for the binding activity observed in the Var-261 cells.

Northern blot analysis using haSR-BI as a probe showed that a single major mRNA (approximately 3.4 kb) was present in similar amounts in both the parental ldlA and Var-261 cells, indicating that the cells express similar amounts of haSR-BI mRNA. Therefore, although haSR-BI encodes a scavenger receptor, it is apparently expressed in both ldlA and Var-261 cells at very low levels. Taken together with the specificity data described above, these results indicate that haSR-BI is a novel scavenger receptor which was not responsible for most of the scavenger receptor activity in Var-261 cells.

#### Analysis of haSR-BI cDNA

The cloned haSR-BI cDNA is approximately 2.9 kb long. The sequences of the 5' untranslated region, the coding region, and a portion of the 3' untranslated region are shown in Sequence Listing ID No. 3. The predicted protein sequence is 509 amino acids (Sequence Listing ID No. 4) with a calculated molecular weight of 57 kD. The murine cDNA was subsequently isolated. The nucleotide sequence is shown in Sequence Listing ID No. 7 and the predicted amino acid sequence is shown in Sequence Listing ID No. 8.

Based on comparison of amino acid sequence, haSR-BI has homology along its entire length to members of the CD36 family of membrane proteins: CD36 (32%, 31% and 33% amino acid identities with the human (Oquendo et al., 1989), murine (Endemann et al., 1993) and rat (also known as "FAT", Abumrad et al., 1993) homologs),

rat LIMPII (33%, a lysosomal integral membrane protein (Vega et al., 1991 *J. Biol. Chem.* 266, 16818-16824), and two *Drosophila melanogaster* proteins, emp (29%, Hart and Wilcox, 1993 *J. Mol. Biol.* 234, 249-253) and  
5 "dCD36" (31%, Genbank #DMCD361). All of these, with the exception of "dCD36", have two internal hydrophobic domains, which have been suggested to serve as membrane spanning domains, and a series of conserved cysteines and putative N-linked  
10 glycosylation sites (see Vega et al., 1991 for a description of the common sequence elements).

*Characterization and comparison of haSR-BI binding with human CD36 binding affinity and selectivity*

15 To further characterize the binding properties of huCD36 and compare them with those of haSR-BI, the properties of COS cells transiently transfected with a cDNA expression vector for human CD36 (obtained from Dr. Brian Seed, Oquendo et al., 1989) were examined.

20 COS cells were transfected on day 1 with a plasmid encoding CD36. On day 2 the transfected cells were set in 6-well dishes at  $1 \times 10^6$  cells/well in medium A plus 1 mM sodium butyrate. On day 3,  $^{125}\text{I}$ -AcLDL binding at 4°C was measured. The high affinity  
25 values shown represent the differences between measurements made in the absence (duplicate incubations) and presence (single incubations) of an excess of the unlabeled competitor M-BSA (200 µg/ml). On day 3, degradation of  $^{125}\text{I}$ -AcLDL (5 µg/ml) was  
30 measured after a 5 hr incubation at 37°C in the absence (duplicate determinations) or presence (duplicate determinations) of the indicated competitors. The values represent the means of four determinations from two identical experiments (the  
35 error bars represent standard deviations). The 100% of control values (ng/5 hr/mg cell protein) from the two experiments were 232 and 103.

Figure 4A shows that expression of huCD36 conferred high affinity  $^{125}\text{I}$ -AcLDL binding on  
40 transiently transfected COS cells at 4°C with an

apparent dissociation constant in the range of approximately 5  $\mu\text{g}$  protein/ml. Figure 4B shows that the receptor activity was inhibited by M-BSA and AcLDL, but not by other polyanions which inhibit class A macrophage scavenger receptors, including fucoidin, poly I, poly G, and ReLPS. Figure 5 is a graph directly comparing lipoprotein inhibition of  $^{125}\text{I}$ -AcLDL binding to haSR-BI and huCD36. The graph clearly demonstrates the similarities: i.e., binding to both receptors is inhibited by the modified LDL; and the differences: only haSR-BI binding is inhibited by LDL.

#### *HDL Binding to haSR-BI*

Further binding studies were conducted to determine if SR-BI bound HDL and VLDL, as well as LDL and anionic phospholipids. Competition binding studies demonstrate that HDL and VLDL (400  $\mu\text{g}/\text{ml}$ ) competitively inhibit binding of  $^{125}\text{I}$ -AcLDL to HaSR-BI, providing further support for the potential role of this receptor in lipoprotein and lipid metabolism. Direct binding of  $^{125}\text{I}$ -HDL to cells expressing SR-BI is shown in Figure 8a. Studies were also conducted using CD36, which demonstrated that HDL bound competitively to CD36.

#### *Phospholipid Binding*

To determine if phospholipids could bind to haSR-BI, 105 nm diameter PS liposomes (PS/phosphatidylcholine/cholesterol, ratio 1:1:1) radiolabeled with trace amounts of  $[^3\text{H}]$ dipalmitoyl phosphatidylcholine (62 Ci/mmol) were prepared and binding at 4°C to untransfected cells (ldlA) and transfected cells which express haSR-BI (ldlA[haSR-BI]) cells determined. The results are shown in Figures 7a, 7b, 7c, 7d, and 7e. Fig 7d shows that there was substantial, high affinity ( $K_d$ -15  $\mu\text{g}$  phospholipid/ml) and saturable binding to the transfected cells, but relatively little binding to the untransfected cells. Assuming that the phospholipid and cholesterol were uniformly distributed in homogenous liposomes

containing approximately 107,400 molecules of phospholipid/liposome, it is estimated that the  $K_d$  (mol of PS liposomes/liter) to be approximately 0.18 nM. PS binding was apparently independent of divalent cations because it was not inhibited by EDTA (1-10 mM). Binding depended on the phospholipid composition of the liposomes. In contrast to that of [ $^3$ H]PS liposomes, the binding of radiolabeled PC liposomes (PC/cholesterol, 2:1) was very low and similar to [ $^3$ H]PS binding to untransfected ldla cells.

These results indicated that phospholipids can bind to haSR-BI and that this binding might depend on the charge of the phospholipid head group. The specificity of the binding was further assessed by determining the competition for [ $^3$ H]PS binding by unlabeled liposomes of various compositions (indicated phospholipid/PC/cholesterol, ratio 1:1:1). Figure 7e shows that the anionic phospholipids PS and PI were effective inhibitors while the zwitterionic PC and PE as well as SM were not. PA, another anionic phospholipid, was able to compete, but not as effectively as PS and PI. Figure 7a shows that PS and PI liposomes inhibited virtually all of the binding of  $^{125}$ I-AcLDL to haSR-BI in transiently transfected COS cells (greater than 50% inhibition at concentrations greater than 10  $\mu$ g/phospholipid/ml), while PC had virtually no effect at concentrations as high as 250  $\mu$ g phospholipid/ml. Similar results are shown for cells transfected with huCD36 in Figure 7b. The extent of PS inhibition of  $^{125}$ I-LDL binding depended on the relative PS content of the liposomes. Figure 7c shows that inhibition by 500  $\mu$ g phospholipid/ml increased substantially as the amount of PS in PS/PC mixed liposomes increased from 0 to 50 mol % of total phospholipid, with greater than 50% inhibition occurring when the PS mol % was greater than 10. These competition experiments suggest that anionic phospholipids bound to haSR-BI at a site close to or identical with the site of native and modified LDL



binding and that polyvalent binding via multiple anionic phospholipid molecules may be involved.

The specific recognition of anionic phospholipids in the outer leaflets of cell membranes and lipoproteins by cell surface receptors may play an important role in a variety of physiologic and pathophysiologic process, including recognition of damaged or senescent cells by the reticuloendothelial system or lipoprotein homeostasis. These studies support the role of SR-BI in these interactions.

*Tissue distribution of haSR-BI*

To explore the physiological functions of haSR-BI, the tissue distribution of haSR-BI was determined in murine tissues and during differentiation of 3T3-L1 cells into adipocytes using Northern blot. Each lane was loaded with 0.5  $\mu$ g of poly(A)+ RNA prepared from the murine tissues: kidney, liver, brain, testis, fat, diaphragm, heart, lung, spleen, or from 3T3-L1 fibroblasts which were either nonconfluent at the fibroblast stage or confluent and induced to differentiate over a period of 0, 2, 4, 6 or 8 days. The blots were hybridized with a 600 base pair fragment of the coding region of haSR-BI. Hsp70 cDNA was used as a control for equal RNA loading. In the Northern of the murine tissues, the same blot was used for both SR-BI and hsp70 hybridizations. In the Northern of the differentiating 3T3-L1 cells, parallel blots were used. The blot of the murine tissues was also hybridized with a CD36 probe demonstrating that CD36 and SR-BI probes recognized different mRNA species.

One predominant band of approximately 2.4 kb was most abundant in fat and was present at moderate levels in lung and liver. There was little expression in the remaining tissues tested, which included kidney, brain, testis, diaphragm, heart, and spleen. To further investigate the expression of SR-BI in fat, the SR-BI mRNA levels in 3T3-L1 cells which were induced to differentiate into adipocytes were

determined. The levels of SR-BI mRNA were found to increase during differentiation in a manner similar to that previously demonstrated for the glucose transporter GLUT4 and for Rab3D, a small molecular weight GTP binding protein (Baldini et al., 1992). These data, together with the data showing that modified LDL binding is inhibited by LDL, indicate that SR-BI plays a physiological role in lipid metabolism in adipocytes. In this regard, it is noteworthy that rat CD36 (also known as the "FAT" protein) was cloned as a result of its ability to directly bind reactive fatty acid esters (Abumrad et al., 1993). CD36 message is also markedly increased upon differentiation of the cultured lines 3T3 F442A and Ob1771 into adipocytes, as is the message for SR-BI in the 3T3-L1 adipocyte system. The expression patterns of SR-BI mRNA and CD36 mRNA are similar, but not identical. Both are found in high levels in adipose tissue. One notable difference in expression levels is found in the liver, where SR-BI expression is moderate but no CD36 message was detected. The observation that native LDL competes for AcLDL binding to SR-BI further indicated that SR-BI may play a role in lipid metabolism.

In contrast to the studies detecting mRNA encoding SR-BI, blots using polyclonal antibodies to a cytoplasmic region of SR-BI found that very high levels of protein were present in liver, adrenal tissues, and ovary in mice and rats, but only very low or undetectable levels in either white or brown fat, muscle or a variety of other tissues. Bands in the rat tissues were present at approximately 80 to 95 kD in liver but a smaller molecular weight protein of approximately 57 to 69 kD was detected in the steroidogenic tissues, including adrenal tissues and testes. This indicates that the mRNA present in the adipose or steroidogenic tissue actually encodes a close relative of SR-BI, rather than SR-BI, that the SR-BI mRNA is not translated into protein in fat in

rodents, and/or that there may be alternative splicing of the SR-BI gene. In the mouse tissues, only the 80 to 95 kD form was observed in the liver and steroidogenic tissues. This is the same form observed  
5 in transfected cultured cells.

*Recycling of HDL and Lipid Uptake Studies*

It is significant that SR-BI binds HDL and is present in high levels in the liver and in tissues which are known to take up cholesterol from HDL for  
10 use in synthesis of steroids. Further studies were conducted to determine if the HDL bound to cells expressing SR-BI is recycled, i.e., if lipid bound to the HDL is taken up by the cell over time while the amount of HDL remains relatively constant. This is  
15 demonstrated in Figure 8b. It is evident that the amount of HDL bound by the cells, as measured using <sup>125</sup>I-labeled HDL, remains relatively constant after a steady state condition is reached thirty minutes to an hour after addition of the HDL to the cells. In  
20 contrast, the amount of fluorescent lipid in the cells continues to increase over time, demonstrating that the HDL must be binding the cells, not being internalized or degraded, and that the lipid bound to the HDL being internalized and retained within the  
25 cells. The results with DiI are predictive of results with cholesterol ester. Controls with untransfected cells, in which there was no binding to the cells or fluorescent lipid transfer, are evidence that this is a receptor dependent function.

30 The possible roles of SR-BI in HDL metabolism is shown schematically in Figure 9. It is extremely likely that SR-BI and the related SR-B proteins play critical roles in HDL mediated lipid metabolism and transport. SR-BI appears to be responsible for  
35 cholesterol delivery to steroidogenic tissues and liver. It would be useful to increase expression of SR-BI in cells in which uptake of cholesterol can be increased, freeing HDL to serve as a means for removal

of cholesterol from storage cells such as foam cells where it can play a role in atherogenesis.

## II. *Drosophila melanogaster* scavenger receptor protein

In an effort to further define the structures and functions of scavenger receptors, receptor expression was investigated in a representative invertebrate, *Drosophila melanogaster* (Abrams, et al. 1993 Proc. Natl. Acad. USA 89:10375-10379). By examining *D. melanogaster* embryos using fluorescently labeled AcLDL and <sup>125</sup>I-AcLDL as probes for receptor activity in vivo and in vitro, fluorescence was found to be distributed throughout the interstitial spaces of the body cavity in a pattern characteristic of the distribution of embryonic macrophages and was observed in cells with multivesicular inclusions characteristic of macrophages. Further analysis of primary embryonic cell cultures showed that uptake was macrophage specific and exhibited the broad specificity of mammalian scavenger receptors.

Two commonly used *Drosophila* cell lines were examined for scavenger receptor activity: L2 and Kc. Only the Schneider L2 cells, not the Kc cells, exhibit a scavenger receptor-mediated endocytic pathway, which is almost identical to that of mammalian macrophages. The L2 cell receptors exhibit characteristic scavenger receptor-like broad polyanion-binding specificity, and mediate high-affinity and saturable binding, uptake and degradation of AcLDL. In L2 cells, the kinetics of intracellular ligand degradation after binding and uptake shows a lag phase, intracellular ligand degradation is chloroquine sensitive, and endocytosis is temperature dependent.

### Preparation of cDNA and genomic libraries:

To facilitate both hybridization and expression cloning, cDNA libraries were generated from poly A+ RNA from L2 cells using standard procedures, as described above for Var-261 cells, and libraries were generated in the expression vector pcDNA1

(Invitrogen). The average insert size for the bidirectional L2 cell library is about 1.4 kb. DNAs for hybridization and transfection experiments were prepared as follows: *E. coli* strain MC1061/P3 is transformed with the cDNA expression library by electroporation and the transformed cells plated on LB-A/T plates (LB with Amp (15 µg/ml)/Tet (8 µg/ml)) to obtain isolated colonies at densities of 2,000 to 10,000 per 150 mm dish. Each plate, representing one pool, is scraped to recover the bacteria and a pool of library plasmids is isolated from each dish of bacteria using a midiprep method (miniprep method of Sambrook et al., 1989 Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, scaled up five to ten fold). The pools can be used for Southern blot analysis for hybridization screening and for transfection into COS or CHO cells for expression cloning.

Expression in cultured mammalian cells.

Expression of the receptors can be detected using either or both fluorescence microscopy and light microscopy of emulsion autoradiographs. Transient expression can be routinely obtained in COS cells, stable expression in CHO cells.

*Fluorescence screening:* Scavenger receptor activity can be detected using fluorescent, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate-labeled AcLDL (DiI-AcLDL). On day 0, COS M6 cells are plated in 35 mm culture dishes (3 to 4 x 10<sup>5</sup> cells/dish) in medium A (DMEM supplemented with 100 units penicillin/ml medium, 100 µg streptomycin/ml medium, and 2 mM glutamine) containing 10% FBS (medium B). On day 1, the cells are transfected with 0.5 µg/dish of library DNA following the method of Cullen, 1987 Methods in Enz. 152:684-704. On day 2, monolayers were re-fed with modified medium B 1 mM sodium butyrate. On day 3, the monolayers are re-fed with modified medium B containing between one and five µg protein/ml of DiI-AcLDL. After a 5 hr incubation

at 37°C, the plates are washed two times with PBS and the cells fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. The presence of fluorescent DiI in the fixed cells is determined by visual  
5 screening using a Lietz inverted fluorescence microscope.

After screening the bidirectional L2 cell library, a pool of approximately 3500 clones which reproducibly conferred DiI-AcLDL endocytic activity in  
10 transiently transfected COS cells was identified. This pool was subdivided into 18 subpools of approximately 350 clones each, which were transfected into COS cells. One of these subpools also conferred DiI-AcLDL endocytic activity.

15 The clone from the subpool was isolated and transfected into CHO cells to yield CHO[dSR-CI]-2.6a cells. The nucleic acid (Sequence ID No. 5) and amino acid (Sequence ID No. 6) sequences were also obtained using the methods described above.

20 Binding Activity of CHO[dSR-CI]-2.6a Cells

Binding of  $^{125}\text{I}$ -AcLDL to CHO[dSR-CI]-2.6a cells was measured as ng AcLDL protein bound/mg cell protein at concentrations of 1, 3, 6, 13, 25, and 50  $\mu\text{g}$  AcLDL protein/ml. Uptake was also measured, comparing  
25 total, nonspecific and specific binding.

As shown by Figure 6A, dSR-CI binds AcLDL. As shown by Figure 6B, AcLDL was also taken up by the cells expressing dSR-CI.

Degradation was measured to confirm that dSR-CI  
30 mediated internalization of the AcLDL. The results shown in Figure 6C confirm the selective degradation of AcLDL.

Competitive binding of AcLDL was performed to more accurately define the specificity of the AcLDL  
35 binding. The results are shown below in Table 1.

**Table 1. Competitive Inhibition of  $^{125}$ I-AcLDL Degradation Activity by CHO[dSR-CI]-2.6a Cells.**

<u>Competitor</u>	<u>Degradation (% No Competitor)</u>
None	100
AcLDL (400 $\mu$ g/ml)	4.8
LDL (400 $\mu$ g/ml)	97.6
M-BSA (400 $\mu$ g/ml)	6.7
BSA (400 $\mu$ g/ml)	94.4
poly I (400 $\mu$ g/ml)	2.7
dA5G37 (100 $\mu$ g/ml)	5.1
dA37 (100 $\mu$ g/ml)	98.2
dA37 (400 $\mu$ g/ml)	43.0
Dextran Sulfate	4.2
Dextran (400 $\mu$ g/ml)	100
Poly D-glutamic acid (400 $\mu$ g/ml)	34

### III. Applications of the Scavenger Receptor Proteins

The presence of scavenger receptors on both mammalian and *Drosophila* macrophages suggests that they mediate critical, well-conserved functions, possibly pathogen recognition, and raises the possibility that they may have appeared early in the evolution of host defense systems. In this regard, it is known that postembryonic macrophage-like hemocytes in *Drosophila* participate in wound healing, encapsulation of pathogens, and phagocytosis. Furthermore, macrophages play an important role in the recognition of apoptotic or senescent cells during the course of development, normal cell turnover, and aging, although it is not known if the scavenger receptors are also involved in these processes. The binding specificity of the SR-BI protein implicates this receptor in additional or alternative roles critical to HDL metabolism and the delivery of cholesterol to steroidogenic tissues.

Accordingly, the understanding of the structure and functions of the receptor proteins described herein, as well as the cDNAs encoding these proteins, have a variety of uses. Specifically, the proteins and their DNAs can be used in screening of drugs which modulate the activity and/or the expression of the receptors; in screening of patient samples for the

presence of functional receptor protein; in the case of the SR-BI receptor protein, removal of LDL, modified LDL, VLDL, or HDL by reaction with immobilized receptor protein; use of the DNA to  
5 construct probes for screening of libraries for other receptors, including the human equivalents, and the regulatory sequences controlling the expression of the other receptors as well as SR-BI and SR-CI. These drugs, when identified, may be useful in treating or  
10 preventing atherosclerosis, fat uptake by adipocytes, and some types of immune disorders.

Isolation of other receptor proteins.

The nucleotide sequences identified herein as encoding hamster SR-BI and *Drosophila melanogaster* SR-CI are useful as probes for screening of libraries for  
15 the presence of related receptors. Libraries are constructed from cells of a desired species, such as humans, which are then screened with all or a portion of the nucleotide sequence encoding either SR-BI or  
20 SR-CI. Specific regions of interest are those portions of the nucleotide sequence which encode regions of the protein conserved between different receptors; between the same receptors from different species; and within discrete regions of the receptor  
25 proteins: the cytoplasmic region, the transmembrane region, the "stem" regions that may include EGF repeats, collagen like regions  $\alpha$ -helical coiled regions, or regions having a high density of cysteines (CCP domains), and specific ligand regions. These  
30 regions are identified by structural analysis such as that which has been used to generate the schematics in Figures 1A and 1B, using methods routinely available to those skilled in the art. These methods include chemical crosslinking, electrophoretic analysis,  
35 hydrodynamic studies, and electron microscopy and computer assisted analysis of structure based on predicted amino acid sequence.

As used herein, unless specifically stated otherwise, the term "SR-BI" refers to the nucleotide



and amino acid sequences, respectively, shown in Sequence ID Nos. 3 and 4, and 7 and 8, and degenerate variants thereof and their equivalents in other species of origin, especially human, as well as  
5 functionally equivalent variants, having additions, deletions, and substitutions of either nucleotides or amino acids which do not significantly alter the functional activity of the protein as a receptor characterized by the binding activity identified  
10 above. The term "SR-CI" refers to the sequences shown in Sequence ID Nos. 5 and 6, and degenerate variants thereof and their equivalents in other species of origin, especially human, as well as functionally  
15 equivalent variants, having additions, deletions, and substitutions of either nucleotides or amino acids which do not significantly alter the functional activity of the protein as a receptor characterized by the binding activity identified above.

Preferred uses for these sequences, especially  
20 those in the Sequence Listings below, are for the cloning of equivalent receptor molecules present in human cells, for the isolation and characterization of the regulatory sequences present in the genome which controlled the extent to which a particular receptor  
25 is expressed in a cell, and for the screening of drugs altering binding of or endocytosis of ligand by the scavenger receptor proteins.

#### Isolation of Receptor Proteins

Additional receptor proteins for study can be  
30 obtained by expression in suitable recombinant host systems, such as mammalian, yeast, bacteria, or insect cells. Isolation can be facilitated by making antibodies to the recombinant protein which are then immobilized on substrates for use in purification of  
35 additional receptors.

As discussed above with regard to tissue distribution, it appears that at least one different but closely related protein is encoded by a nucleotide sequence present in steroidogenic tissues in rodents

which hybridizes to Sequence ID No. 3 under stringent conditions, and can be isolated using routine techniques and the materials described herein.

5       Screening of patient samples for expression of receptor proteins.

The sequences disclosed herein are useful in screening of patient samples for the presence of normal receptor proteins, using hybridization assays of patient samples, including blood and tissues.

10      Screening can also be accomplished using antibodies, typically labeled with a fluorescent, radiolabeled, or enzymatic label, or by isolation of target cells and screening for binding activity, as described in the examples above. Typically, one would be screening for

15      expression on either a qualitative or quantitative basis, and for expression of functional receptor.

*Hybridization Probes*

Reaction conditions for hybridization of an oligonucleotide probe or primer to a nucleic acid

20      sequence vary from oligonucleotide to oligonucleotide, depending on factors such as oligonucleotide length, the number of G and C nucleotides, and the composition of the buffer utilized in the hybridization reaction. Moderately stringent hybridization conditions are

25      generally understood by those skilled in the art as conditions approximately 25°C below the melting temperature of a perfectly base-paired double-stranded DNA. Higher specificity is generally achieved by

30      employing incubation conditions having higher temperatures, in other words more stringent conditions. In general, the longer the sequence or higher the G and C content, the higher the temperature and/or salt concentration required. Chapter 11 of the well-known laboratory manual of Sambrook et al.,

35      MOLECULAR CLONING: A LABORATORY MANUAL, second edition, Cold Spring Harbor Laboratory Press, New York (1990) (which is incorporated by reference herein), describes hybridization conditions for oligonucleotide probes and primers in great detail, including a description

of the factors involved and the level of stringency necessary to guarantee hybridization with specificity.

The preferred size of a hybridization probe is from 10 nucleotides to 100,000 nucleotides in length.

5 Below 10 nucleotides, hybridized systems are not stable and will begin to denature above 20°C. Above 100,000 nucleotides, one finds that hybridization (renaturation) becomes a much slower and incomplete process, as described in greater detail in the text  
10 MOLECULAR GENETICS, Stent, G.S. and R. Calender, pp. 213-219 (1971). Ideally, the probe should be from 20 to 10,000 nucleotides. Smaller nucleotide sequences (20-100) lend themselves to production by automated organic synthetic techniques. Sequences from 100-  
15 10,000 nucleotides can be obtained from appropriate restriction endonuclease treatments. The labeling of the smaller probes with the relatively bulky chemiluminescent moieties may in some cases interfere with the hybridization process.

20 *Generation of Antibodies for Diagnostic or Therapeutic Use*

Antibodies to the receptor proteins can also be generated which are useful in detection, characterization or isolation of receptor proteins, as  
25 well as for modifying receptor protein activity, in most cases, through inhibition of binding. Antibodies are generated by standard techniques, using human or animal receptor proteins. Since the proteins exhibit high evolutionary conservation, it may be advantageous  
30 to generate antibodies to a protein of a different species of origin than the species in which the antibodies are to be tested or utilized, looking for those antibodies which are immunoreactive with the most evolutionarily conserved regions. Antibodies are  
35 typically generated by immunization of an animal using an adjuvant such as Freund's adjuvant in combination with an immunogenic amount of the protein administered over a period of weeks in two to three week intervals, then isolated from the serum, or used to make  
40 hybridomas which express the antibodies in culture.

Because the methods for immunizing animals yield antibody which is not of human origin, the antibodies could elicit an adverse effect if administered to humans. Methods for "humanizing" antibodies, or  
5 generating less immunogenic fragments of non-human antibodies, are well known. A humanized antibody is one in which only the antigen-recognized sites, or complementarily-determining hypervariable regions (CDRs) are of non-human origin, whereas all framework  
10 regions (FR) of variable domains are products of human genes. These "humanized" antibodies present a lesser xenographic rejection stimulus when introduced to a human recipient.

To accomplish humanization of a selected mouse  
15 monoclonal antibody, the CDR grafting method described by Daugherty, et al., 1991 Nucl. Acids Res., 19:2471-2476, incorporated herein by reference, may be used. Briefly, the variable region DNA of a selected animal recombinant anti-idiotypic ScFv is sequenced by the  
20 method of Clackson, T., et al., 1991 Nature, 352:624-688, incorporated herein by reference. Using this sequence, animal CDRs are distinguished from animal framework regions (FR) based on locations of the CDRs in known sequences of animal variable genes. Kabat,  
25 H.A., et al., Sequences of Proteins of Immunological Interest, 4th Ed. (U.S. Dept. Health and Human Services, Bethesda, MD, 1987). Once the animal CDRs and FR are identified, the CDRs are grafted onto human heavy chain variable region framework by the use of  
30 synthetic oligonucleotides and polymerase chain reaction (PCR) recombination. Codons for the animal heavy chain CDRs, as well as the available human heavy chain variable region framework, are built in four (each 100 bases long) oligonucleotides. Using PCR, a  
35 grafted DNA sequence of 400 bases is formed that encodes for the recombinant animal CDR/human heavy chain FR protection.

The immunogenic stimulus presented by the monoclonal antibodies so produced may be further

decreased by the use of Pharmacia's (Pharmacia LKB Biotechnology, Sweden) "Recombinant Phage Antibody System" (RPAS), which generates a single-chain Fv fragment (ScFv) which incorporates the complete antigen-binding domain of the antibody. In the RPAS, antibody variable heavy and light chain genes are separately amplified from the hybridoma mRNA and cloned into an expression vector. The heavy and light chain domains are co-expressed on the same polypeptide chain after joining with a short linker DNA which codes for a flexible peptide. This assembly generates a single-chain Fv fragment (ScFv) which incorporates the complete antigen-binding domain of the antibody. Compared to the intact monoclonal antibody, the recombinant ScFv includes a considerably lower number of epitopes, and thereby presents a much weaker immunogenic stimulus when injected into humans.

The antibodies can be formulated in standard pharmaceutical carriers for administration to patients in need thereof. These include saline, phosphate buffered saline, and other aqueous carriers, and liposomes, polymeric microspheres and other controlled release delivery devices, as are well known in the art. The antibodies can also be administered with adjuvant, such as muramyl dipeptide or other materials approved for use in humans (Freund's adjuvant can be used for administration of antibody to animals).

Screening for drugs modifying or altering the extent of receptor function or expression

The receptor proteins are useful as targets for compounds which turn on, or off, or otherwise regulate binding to these receptors. The assays described above clearly provide routine methodology by which a compound can be tested for an inhibitory effect on binding of a specific compound, such as a radiolabeled modified LDL or polyion. The *in vitro* studies of compounds which appear to inhibit binding selectively to the receptors are then confirmed by animal testing. Since the molecules are so highly evolutionarily conserved, it is possible to conduct studies in

laboratory animals such as mice to predict the effects in humans.

Studies based on inhibition of binding are predictive for indirect effects of alteration of receptor binding. For example, inhibition of LDL binding to the SR-BI receptor leads to decreased uptake by cells of LDL and therefore decreases deposition of LDL in cells; similar effects should be observed for inhibition of lipoprotein and/or lipid by adipocytes expressing the SR-BI receptor. Conversely, increasing LDL binding to cells increases removal of lipids from the blood stream and thereby decreases lipid deposition within the blood stream. Studies have been conducted using a stimulator to enhance macrophage uptake of cholesterol and thereby treat atherogenesis, using M-CSF (Schaub, et al., 1994 Arterioscler. Thromb. 14(1), 70-76; Inaba, et al., 1993 J. Clin. Invest. 92(2), 750-757). Although the target of the stimulator is not known with specificity, this provides further support for the rationale for believing the indirect *in vivo* effects can be achieved based on the *in vitro* binding data.

Studies described above and the results shown in Figure 8 demonstrate that drugs increasing expression of SR-BI or closely related proteins in tissues such as liver would be useful in enhancing removal of cholesterol from the circulation and foam cells.

Assays for testing compounds for useful activity can be based solely on interaction with the receptor protein, preferably expressed on the surface of transfected cells such as those described above, although proteins in solution or immobilized on inert substrates can also be utilized, where the indication is inhibition or increase in binding of LDL or modified LDL.

Alternatively, the assays can be based on interaction with the gene sequence encoding the receptor protein, preferably the regulatory sequences directing expression of the receptor protein. For

example, antisense which binds to the regulatory sequences, and/or to the protein encoding sequences can be synthesized using standard oligonucleotide synthetic chemistry. The antisense can be stabilized  
5 for pharmaceutical use using standard methodology (encapsulation in a liposome or microsphere; introduction of modified nucleotides that are resistant to degradation or groups which increase resistance to endonucleases, such as  
10 phosphorothiodates and methylation), then screened initially for alteration of receptor activity in transfected or naturally occurring cells which express the receptor, then *in vivo* in laboratory animals. Typically, the antisense would inhibit expression.  
15 However, sequences which block those sequences which "turn off" synthesis can also be targeted.

The receptor protein for study can be isolated from either naturally occurring cells or cells which have been genetically engineered to express the  
20 receptor, as described in the examples above. In the preferred embodiment, the cells would have been engineered using the intact gene.

*Random generation of receptor or receptor encoding sequence binding molecules.*

25 Molecules with a given function, catalytic or ligand-binding, can be selected for from a complex mixture of random molecules in what has been referred to as "in vitro genetics" (Szostak, TIBS 19:89, 1992). One synthesizes a large pool of molecules bearing  
30 random and defined sequences and subjects that complex mixture, for example, approximately  $10^{15}$  individual sequences in 100  $\mu$ g of a 100 nucleotide RNA, to some selection and enrichment process. For example, by repeated cycles of affinity chromatography and PCR  
35 amplification of the molecules bound to the ligand on the column, Ellington and Szostak (1990) estimated that 1 in  $10^{10}$  RNA molecules folded in such a way as to bind a given ligand. DNA molecules with such ligand-binding behavior have been isolated (Ellington  
40 and Szostak, 1992; Bock et al, 1992).

*Computer assisted drug design*

Computer modeling technology allows visualization of the three-dimensional atomic structure of a selected molecule and the rational design of new compounds that will interact with the molecule. The three-dimensional construct typically depends on data from x-ray crystallographic analyses or NMR imaging of the selected molecule. The molecular dynamics require force field data. The computer graphics systems enable prediction of how a new compound will link to the target molecule and allow experimental manipulation of the structures of the compound and target molecule to perfect binding specificity. Prediction of what the molecule-compound interaction will be when small changes are made in one or both requires molecular mechanics software and computationally intensive computers, usually coupled with user-friendly, menu-driven interfaces between the molecular design program and the user.

Examples of molecular modelling systems are the CHARMM and QUANTA programs, Polygen Corporation, Waltham, MA. CHARMM performs the energy minimization and molecular dynamics functions. QUANTA performs the construction, graphic modelling and analysis of molecular structure. QUANTA allows interactive construction, modification, visualization, and analysis of the behavior of molecules with each other.

A number of articles review computer modeling of drugs interactive with specific proteins, such as Rotivinen, et al., 1988 Acta Pharmaceutica Fennica 97, 159-166; Ripka, New Scientist 54-57 (June 16, 1988); McKinally and Rossmann, 1989 Annu. Rev. Pharmacol. Toxicol. 29, 111-122; Perry and Davies, QSAR: Quantitative Structure-Activity Relationships in Drug Design pp. 189-193 (Alan R. Liss, Inc. 1989); Lewis and Dean, 1989 Proc. R. Soc. Lond. 236, 125-140 and 141-162; and, with respect to a model receptor for nucleic acid components, Askew, et al., 1989 J. Am. Chem. Soc. 111, 1082-1090. Other computer programs



that screen and graphically depict chemicals are available from companies such as BioDesign, Inc., Pasadena, CA., Allelix, Inc., Mississauga, Ontario, Canada, and Hypercube, Inc., Cambridge, Ontario.

- 5 Although these are primarily designed for application to drugs specific to particular proteins, they can be adapted to design of drugs specific to regions of DNA or RNA, once that region is identified.

Although described above with reference to design  
10 and generation of compounds which could alter binding, one could also screen libraries of known compounds, including natural products or synthetic chemicals, and biologically active materials, including proteins, for compounds which are inhibitors or activators.

15 *Generation of nucleic acid regulators*

- Nucleic acid molecules containing the 5' regulatory sequences of the receptor genes can be used to regulate or inhibit gene expression *in vivo*. Vectors, including both plasmid and eukaryotic viral  
20 vectors, may be used to express a particular recombinant 5' flanking region-gene construct in cells depending on the preference and judgment of the skilled practitioner (see, e.g., Sambrook et al., Chapter 16). Furthermore, a number of viral and  
25 nonviral vectors are being developed that enable the introduction of nucleic acid sequences *in vivo* (see, e.g., Mulligan, 1993 Science, 260, 926-932; United States Patent No. 4,980,286; United States Patent No. 4,868,116; incorporated herein by reference).
- 30 Recently, a delivery system was developed in which nucleic acid is encapsulated in cationic liposomes which can be injected intravenously into a mammal. This system has been used to introduce DNA into the cells of multiple tissues of adult mice, including  
35 endothelium and bone marrow (see, e.g., Zhu et al., 1993 Science 261, 209-211; incorporated herein by reference).

The 5' flanking sequences of the receptor gene can also be used to inhibit the expression of the

receptor. For example, an antisense RNA of all or a portion of the 5' flanking region of the receptor gene can be used to inhibit expression of the receptor in vivo. Expression vectors (e.g., retroviral expression  
5 vectors) are already available in the art which can be used to generate an antisense RNA of a selected DNA sequence which is expressed in a cell (see, e.g., U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286). Accordingly, DNA containing all or a portion of the  
10 sequence of the 5' flanking region of the receptor gene can be inserted into an appropriate expression vector so that upon passage into the cell, the transcription of the inserted DNA yields an antisense RNA that is complementary to the mRNA transcript of  
15 the receptor protein gene normally found in the cell. This antisense RNA transcript of the inserted DNA can then base-pair with the normal mRNA transcript found in the cell and thereby prevent the mRNA from being translated. It is of course necessary to select  
20 sequences of the 5' flanking region that are downstream from the transcriptional start sites for the receptor protein gene to ensure that the antisense RNA contains complementary sequences present on the mRNA. Antisense RNA can be generated in vitro  
25 also, and then inserted into cells. Oligonucleotides can be synthesized on an automated synthesizer (e.g., Model 8700 automated synthesizer of Milligen-Biosearch, Burlington, MA or ABI Model 380B). In addition, antisense deoxyoligonucleotides have been  
30 shown to be effective in inhibiting gene transcription and viral replication (see e.g., Zamecnik et al., 1978 Proc. Natl. Acad. Sci. USA 75, 280-284; Zamecnik et al., 1986 Proc. Natl. Acad. Sci., 83, 4143-4146; Wickstrom et al., 1988 Proc. Natl. Acad. Sci. USA 85,  
35 1028-1032; Crooke, 1993 FASEB J. 7, 533-539. Furthermore, recent work has shown that improved inhibition of expression of a gene by antisense oligonucleotides is possible if the antisense oligonucleotides contain modified nucleotides (see,

e.g., Offensperger et. al., 1993 EMBO J. 12, 1257-1262 (in vivo inhibition of duck hepatitis B viral replication and gene expression by antisense phosphorothioate oligodeoxynucleotides); Rosenberg et al., PCT WO 93/01286 (synthesis of sulfurthioate oligonucleotides); Agrawal et al., 1988 Proc. Natl. Acad. Sci. USA 85, 7079-7083 (synthesis of antisense oligonucleoside phosphoramidates and phosphorothioates to inhibit replication of human immunodeficiency virus-1); Sarin et al., 1989 Proc. Natl. Acad. Sci. USA 85, 7448-7794 (synthesis of antisense methylphosphonate oligonucleotides); Shaw et al., 1991 Nucleic Acids Res 19, 747-750 (synthesis of 3' exonuclease-resistant oligonucleotides containing 3' terminal phosphoroamidate modifications); incorporated herein by reference).

The sequences of the 5' flanking region of receptor protein gene can also be used in triple helix (triplex) gene therapy. Oligonucleotides complementary to gene promoter sequences on one of the strands of the DNA have been shown to bind promoter and regulatory sequences to form local triple nucleic acid helices which block transcription of the gene (see, e.g., 1989 Maher et al., Science 245, 725-730; Orson et al., 1991 Nucl. Acids Res. 19, 3435-3441; Postal et al., 1991 Proc. Natl. Acad. Sci. USA 88, 8227-8231; Cooney et al., 1988 Science 241, 456-459; Young et al., 1991 Proc. Natl. Acad. Sci. USA 88, 10023-10026; Duval-Valentin et al., 1992 Proc. Natl. Acad. Sci. USA 89, 504-508; 1992 Blume et al., Nucl. Acids Res. 20, 1777-1784; 1992 Grigoriev et al., J. Biol. Chem. 267, 3389-3395.

Recently, both theoretical calculations and empirical findings have been reported which provide guidance for the design of oligonucleotides for use in oligonucleotide-directed triple helix formation to inhibit gene expression. For example, oligonucleotides should generally be greater than 14 nucleotides in length to ensure target sequence

specificity (see, e.g., Maher et al., (1989); Grigoriev et al., (1992)). Also, many cells avidly take up oligonucleotides that are less than 50 nucleotides in length (see e.g., Orson et al., (1991);

5 Holt et al., 1988 Mol. Cell. Biol. 8, 963-973; Wickstrom et al., 1988 Proc. Natl. Acad. Sci. USA 85, 1028-1032). To reduce susceptibility to intracellular degradation, for example by 3' exonucleases, a free amine can be introduced to a 3' terminal hydroxyl

10... group of oligonucleotides without loss of sequence binding specificity (Orson et al., 1991). Furthermore, more stable triplexes are formed if any cytosines that may be present in the oligonucleotide are methylated, and also if an intercalating agent,

15 such as an acridine derivative, is covalently attached to a 5' terminal phosphate (e.g., via a pentamethylene bridge); again without loss of sequence specificity (Maher et al., (1989); Grigoriev et al., (1992)).

Methods to produce or synthesize oligonucleotides

20 are well known in the art. Such methods can range from standard enzymatic digestion followed by nucleotide fragment isolation (see e.g., Sambrook et al., Chapters 5, 6) to purely synthetic methods, for example, by the cyanoethyl phosphoramidite method

25 using a Milligen or Beckman System 1Plus DNA synthesizer (see also, Ikuta et al., in Ann. Rev. Biochem. 1984 53, 323-356 (phosphotriester and phosphite-triester methods); Narang et al., in Methods Enzymol., 65, 610-620 (1980) (phosphotriester method).

30 Accordingly, DNA sequences of the 5' flanking region of the receptor protein gene described herein can be used to design and construct oligonucleotides including a DNA sequence consisting essentially of at least 15 consecutive nucleotides, with or without base

35 modifications or intercalating agent derivatives, for use in forming triple helices specifically within the 5' flanking region of a receptor protein gene in order to inhibit expression of the gene.

In some cases it may be advantageous to insert enhancers or multiple copies of the regulatory sequences into an expression system to facilitate screening of methods and reagents for manipulation of expression.

#### *Preparation of Receptor Protein Fragments*

Compounds which are effective for blocking binding of the receptor can also consist of fragments of the receptor proteins, expressed recombinantly and cleaved by enzymatic digest or expressed from a sequence encoding a peptide of less than the full length receptor protein. These will typically be soluble proteins, i.e., not including the transmembrane and cytoplasmic regions, although smaller portions determined in the assays described above to inhibit or compete for binding to the receptor proteins can also be utilized. It is a routine matter to make appropriate receptor protein fragments, test for binding, and then utilize. The preferred fragments are of human origin, in order to minimize potential immunological response. The peptides can be as short as five to eight amino acids in length and are easily prepared by standard techniques. They can also be modified to increase *in vivo* half-life, by chemical modification of the amino acids or by attachment to a carrier molecule or inert substrate. Based on studies with other peptide fragments blocking receptor binding, the  $IC_{50}$ , the dose of peptide required to inhibit binding by 50%, ranges from about 50  $\mu M$  to about 300  $\mu M$ , depending on the peptides. These ranges are well within the effective concentrations for the *in vivo* administration of peptides, based on comparison with the RGD-containing peptides, described, for example, in U.S. Patent No. 4,792,525 to Ruoslahti, et al., used *in vivo* to alter cell attachment and phagocytosis. The peptides can also be conjugated to a carrier protein such as keyhole limpet hemocyanin by its N-terminal cysteine by standard procedures such as

the commercial Imject kit from Pierce Chemicals or expressed as a fusion protein, which may have increased efficacy. As noted above, the peptides can be prepared by proteolytic cleavage of the  
5 receptor proteins, or, preferably, by synthetic means. These methods are known to those skilled in the art. An example is the solid phase synthesis described by J. Merrifield, 1964 J. Am. Chem. Soc. 85, 2149, used in U.S. Patent No. 4,792,525, and described in U.S.  
10 Patent No. 4,244,946, wherein a protected alpha-amino acid is coupled to a suitable resin, to initiate synthesis of a peptide starting from the C-terminus of the peptide. Other methods of synthesis are described in U.S. Patent No. 4,305,872 and 4,316,891. These  
15 methods can be used to synthesize peptides having identical sequence to the receptor proteins described herein, or substitutions or additions of amino acids, which can be screened for activity as described above.

The peptide can also be administered as a  
20 pharmaceutically acceptable acid- or base- addition salt, formed by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as formic  
25 acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium hydroxide, potassium hydroxide,  
30 and organic bases such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines.

Peptides containing cyclopropyl amino acids, or amino acids derivatized in a similar fashion, can also be used. These peptides retain their original  
35 activity but have increased half-lives in vivo. Methods known for modifying amino acids, and their use, are known to those skilled in the art, for

example, as described in U.S. Patent No. 4,629,784 to Stammer.

The peptides are generally active when administered parenterally in amounts above about 1  
5  $\mu\text{g/kg}$  of body weight. Based on extrapolation from other proteins, for treatment of most inflammatory disorders, the dosage range will be between 0.1 to 70 mg/kg of body weight. This dosage will be dependent, in part, on whether one or more peptides are  
10 administered.

#### *Pharmaceutical Compositions*

Compounds which alter receptor protein binding are preferably administered in a pharmaceutically acceptable vehicle. Suitable pharmaceutical vehicles  
15 are known to those skilled in the art. For parenteral administration, the compound will usually be dissolved or suspended in sterile water or saline. For enteral administration, the compound will be incorporated into an inert carrier in tablet, liquid,  
20 or capsular form. Suitable carriers may be starches or sugars and include lubricants, flavorings, binders, and other materials of the same nature. The compounds can also be administered locally by topical application of a solution, cream, gel, or polymeric  
25 material (for example, a Pluronic™, BASF).

Alternatively, the compound may be administered in liposomes or microspheres (or microparticles). Methods for preparing liposomes and microspheres for administration to a patient are known to those skilled  
30 in the art. U.S. Patent No. 4,789,734 describe methods for encapsulating biological materials in liposomes. Essentially, the material is dissolved in an aqueous solution, the appropriate phospholipids and lipids added, along with surfactants if required, and  
35 the material dialyzed or sonicated, as necessary. A review of known methods is by G. Gregoriadis, Chapter 14. "Liposomes", Drug Carriers in Biology and Medicine pp. 287-341 (Academic Press, 1979). Microspheres formed of polymers or proteins are well

known to those skilled in the art, and can be tailored for passage through the gastrointestinal tract directly into the bloodstream. Alternatively, the compound can be incorporated and the microspheres, or  
5 composite of microspheres, implanted for slow release over a period of time, ranging from days to months. See, for example, U.S. Patent No. 4,906,474, 4,925,673, and 3,625,214.

Removal of LDL from patients or patient samples

10 The SR-BI receptor proteins can be used to remove LDL from patient blood, by immobilizing the receptor on a suitable substrate, such as the cellulose membrane of a dialysis unit, using conventional coupling, for example, using carboimide. The  
15 patient's blood is then dialyzed through the unit.

Modifications and variations of the methods and materials described herein will be obvious to those skilled in the art and are intended to be encompassed by the following claims. The teachings of the  
20 references cited herein are specifically incorporated herein.



## SEQUENCE LISTING

- (1) GENERAL INFORMATION:
- (i) APPLICANT: Massachusetts Institute of Technology
  - (ii) TITLE OF INVENTION: Class BI and CI Scavenger Receptors
  - (iii) NUMBER OF SEQUENCES: 8
  - (iv) CORRESPONDENCE ADDRESS:
    - (A) ADDRESSEE: Patrea L. Pabst
    - (B) STREET: 2800 One Atlantic Center  
1201 West Peachtree Street
    - (C) CITY: Atlanta
    - (D) STATE: Georgia
    - (E) COUNTRY: USA
    - (F) ZIP: 30309-3450
  - (v) COMPUTER READABLE FORM:
    - (A) MEDIUM TYPE: Floppy disk
    - (B) COMPUTER: IBM PC compatible
    - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
    - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
  - (vi) CURRENT APPLICATION DATA:
    - (A) APPLICATION NUMBER:
    - (B) FILING DATE:
    - (C) CLASSIFICATION:
  - (viii) ATTORNEY/AGENT INFORMATION:
    - (A) NAME: Pabst, Patrea L.
    - (B) REGISTRATION NUMBER: 31,284
    - (C) REFERENCE/DOCKET NUMBER: MIT6620
  - (ix) TELECOMMUNICATION INFORMATION:
    - (A) TELEPHONE: (404) 873-8794
    - (B) TELEFAX: (404) 873-8795
- (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 20 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: DNA
  - (iii) HYPOTHETICAL: NO
  - (iv) ANTI-SENSE: NO
  - (x) PUBLICATION INFORMATION:
    - (A) AUTHORS: Ashkenas, et al.
    - (C) JOURNAL: J. Lipid Res.
    - (D) VOLUME: 34
    - (F) PAGES: 983-1000
    - (G) DATE: 1993
    - (K) RELEVANT RESIDUES IN SEQ ID NO:1: FROM 1 TO 20
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AATGAAGAAC TGCTTAGTTT

20

- (2) INFORMATION FOR SEQ ID NO:2:
- (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 18 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: DNA
  - (iii) HYPOTHETICAL: NO
  - (iv) ANTI-SENSE: NO
  - (x) PUBLICATION INFORMATION:
    - (A) AUTHORS: Ashkenas, et al.
    - (C) JOURNAL: J. Lipid Res.
    - (D) VOLUME: 34
    - (F) PAGES: 983-1000
    - (G) DATE: 1993
    - (K) RELEVANT RESIDUES IN SEQ ID NO:1: FROM 1 TO 18
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AATCAAGGAA TTAACTG

18

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1788 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (iii) HYPOTHETICAL: NO

## (iv) ANTI-SENSE: NO

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 156..1683
- (D) OTHER INFORMATION: /function= "Nucleotides 156 through 1683 encode the amino acid sequence for the Hamster Scavenger Receptor Class B-I."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```

GCCACCTGCA GGGCTACTGC TGCTCCGGCC ACTGCCTGAG ACTCACCTTG CTGGAACGTG      60
AGCCTCGGCT TCTGTCATCT CTGTGGCCTC TGTCGCTTCT GTCGCTGTCC CCCTTCAGTC      120
CCTGAGCCCC GCGAGCCCGG GCCGCACACG CGGACATGGG CGGCAGCGCC AGGGCGCGCT      180
GGGTGGCGGT GGGGCTGGGC GTCGTGGGGC TGCTGTGCGC TGTGCTCGGT GTGGTTATGA      240
TCCTCGTGAT GCCCTCGCTC ATCAAACAGC AGGTACTGAA GAATGTCCGC ATAGACCCCA      300
GCAGCCTGTC CTTTGCAATG TGAAGGAGA TCCCTGTACC CTTCTACTTG TCCGTCTACT      360
TCTTCGAGGT GGTCAATCCC AGCGAGATCC TAAAGGGTGA GAAGCCAGTA GTGCGGGAGC      420
GTGGACCCTA TGTCTACAGG GAATTCAGAC ATAAGGCCAA CATCACCTTC AATGACAATG      480
ATACTGTGTC CTTTGTGGAG CACCGCAGCC TCCATTTCCA GCCGGACAGG TCCCACGGCT      540
CTGAGAGTGA CTACATTATA CTGCCTAACA TTCTGGTCTT GGGGGGCGCA GTAATGATGG      600
AGAGCAAGTC TGCAGGCCCTG AAGCTGATGA TGACCTTGGG GCTGGCCACC TTGGGCCAGC      660
GTGCCTTTAT GAACCGAACA GTTGGTGAGA TCCTGTGGGG CTATGAGGAT CCCTTCGTGA      720
ATTTTATCAA CAAATACTTA CCAGACATGT TCCCCATCAA GGGCAAGTTC GGCCTGTTTG      780
TTGAGATGAA CAACTCAGAC TCTGGGCTCT TCACTGTGTT CACGGGCGTC CAGAACTTCA      840
GCAAGATCCA CCTGGTGGAC AGATGGAATG GGCTCAGCAA GGTCAACTAC TGGCATTGAG      900
AGCAGTGCAA CATGATCAAT GGCACCTCCG GGCAGATGTG GGCACCATTG ATGACACCCC      960
AGTCCTCGCT GGAATTCTTC AGTCCGAAG CCTGCAGGTC TATGAAGCTC ACCTACCATG     1020
ATTCAGGGGT GTTTGAAGGC ATCCCCACCT ATCGCTTAC AGCCCCTAAA ACTTTGTTTG     1080
CCAATGGGTC TGTTTACCCA CCAATGAAG GTTTCTGCCC GTGCCTTGAA TCCGGCATTC     1140
AAAATGTCAG CACTTGCAGG TTTGGTGCAC CCCTGTTTCT GTCACACCCT CACTTCTACA     1200
ATGCAGACCC TGTGCTATCA GAAGCCGTTT TGGGTCTGAA CCCTGACCCA AGGGAGCATT     1260
CTTTGTTCCT TGACATCCAT CCGGTCACTG GGATCCCCAT GAACTGTTCT GTGAAGTTGC     1320
AGATAAGCCT CTACATCAAA GCTGTCAAGG GCATTGGGCA AACAGGGAAG ATCGAGCCCC     1380
TGGTCCTCCC ATTGCTGTGG TTTGAGCAGA GCGGTGCCAT GGGCGGCGAG CCCCTGAACA     1440
CGTTCTACAC GCAGCTGGTG CTGATGCCCC AGGTACTTCA GTATGTGCAG TATGTGCTGC     1500
TGGGGCTGGG CGGCCTCCTG CTGCTGGTGC CCGTCATCTA CCAGTTGCGC AGCCAGGAGA     1560

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AATGCTTTTT ATTTTGGAGT GGTAGTAAAA AGGGCTCGCA GGATAAGGAG GCCATTCAGG 1620  
 CCTACTCTGA GTCTCTGATG TCACCAGCTG CCAAGGGCAC GGTGCTGCAA GAAGCCAAGC 1680  
 TGTAGGGTCC CAAAGACACC ACGAGCCCCC CCAACCTGAT AGCTTGGTCA GACCAGCCAT 1740  
 CCAGCCCCTA CACCCCGCTT CTTGAGGACT CTCTCAGCGG ACAGTCGC 1788

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 509 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (v) FRAGMENT TYPE: internal
- (ix) FEATURE:
  - (A) NAME/KEY: misc\_feature
  - (B) LOCATION: 1..509
  - (D) OTHER INFORMATION: /function= "Amino acid sequence for the Hamster Scavenger Receptor Class B-I."
- (ix) FEATURE:
  - (A) NAME/KEY: Domain
  - (B) LOCATION: 9..32
  - (D) OTHER INFORMATION: /note= "Putative transmembrane domain."
- (ix) FEATURE:
  - (A) NAME/KEY: Domain
  - (B) LOCATION: 440..464
  - (D) OTHER INFORMATION: /note= "Putative transmembrane domain."
- (ix) FEATURE:
  - (A) NAME/KEY: Modified-site
  - (B) LOCATION: 1..385
  - (D) OTHER INFORMATION: /note= "Positions 102-104, 108-110, 173-175, 212-214, 227-229, 255-257, 310-312, 330-332 and 383-385 represent potential N-linked glycosylation sites."
- (ix) FEATURE:
  - (A) NAME/KEY: Modified-site
  - (B) LOCATION: 21..470
  - (D) OTHER INFORMATION: /note= "The cysteines at positions 21, 251, 280, 321, 323, 334, 384 and 470 represent potential disulfide linkages."
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Gly Gly Ser Ala Arg Ala Arg Trp Val Ala Val Gly Leu Gly Val  
 1 5 10 15  
 Val Gly Leu Leu Cys Ala Val Leu Gly Val Val Met Ile Leu Val Met  
 20 25 30  
 Pro Ser Leu Ile Lys Gln Gln Val Leu Lys Asn Val Arg Ile Asp Pro  
 35 40 45  
 Ser Ser Leu Ser Phe Ala Met Trp Lys Glu Ile Pro Val Pro Phe Tyr  
 50 55 60  
 Leu Ser Val Tyr Phe Phe Glu Val Val Asn Pro Ser Glu Ile Leu Lys  
 65 70 75 80  
 Gly Glu Lys Pro Val Val Arg Glu Arg Gly Pro Tyr Val Tyr Arg Glu  
 85 90 95  
 Phe Arg His Lys Ala Asn Ile Thr Phe Asn Asp Asn Asp Thr Val Ser  
 100 105 110  
 Phe Val Glu His Arg Ser Leu His Phe Gln Pro Asp Arg Ser His Gly  
 115 120 125

Ser Glu Ser Asp Tyr Ile Ile Leu Pro Asn Ile Leu Val Leu Gly Gly  
 130 135 140  
 Ala Val Met Met Glu Ser Lys Ser Ala Gly Leu Lys Leu Met Met Thr  
 145 150 155 160  
 Leu Gly Leu Ala Thr Leu Gly Gln Arg Ala Phe Met Asn Arg Thr Val  
 165 170 175  
 Gly Glu Ile Leu Trp Gly Tyr Glu Asp Pro Phe Val Asn Phe Ile Asn  
 180 185 190  
 Lys Tyr Leu Pro Asp Met Phe Pro Ile Lys Gly Lys Phe Gly Leu Phe  
 195 200 205  
 Val Glu Met Asn Asn Ser Asp Ser Gly Leu Phe Thr Val Phe Thr Gly  
 210 215 220  
 Val Gln Asn Phe Ser Lys Ile His Leu Val Asp Arg Trp Asn Gly Leu  
 225 230 235 240  
 Ser Lys Val Asn Tyr Trp His Ser Glu Gln Cys Asn Met Ile Asn Gly  
 245 250 255  
 Thr Ser Gly Gln Met Trp Ala Pro Phe Met Thr Pro Gln Ser Ser Leu  
 260 265 270  
 Glu Phe Phe Ser Pro Glu Ala Cys Arg Ser Met Lys Leu Thr Tyr His  
 275 280 285  
 Asp Ser Gly Val Phe Glu Gly Ile Pro Thr Tyr Arg Phe Thr Ala Pro  
 290 295 300  
 Lys Thr Leu Phe Ala Asn Gly Ser Val Tyr Pro Pro Asn Glu Gly Phe  
 305 310 315 320  
 Cys Pro Cys Leu Glu Ser Gly Ile Gln Asn Val Ser Thr Cys Arg Phe  
 325 330 335  
 Gly Ala Pro Leu Phe Leu Ser His Pro His Phe Tyr Asn Ala Asp Pro  
 340 345 350  
 Val Leu Ser Glu Ala Val Leu Gly Leu Asn Pro Asp Pro Arg Glu His  
 355 360 365  
 Ser Leu Phe Leu Asp Ile His Pro Val Thr Gly Ile Pro Met Asn Cys  
 370 375 380  
 Ser Val Lys Leu Gln Ile Ser Leu Tyr Ile Lys Ala Val Lys Gly Ile  
 385 390 395 400  
 Gly Gln Thr Gly Lys Ile Glu Pro Val Val Leu Pro Leu Leu Trp Phe  
 405 410 415  
 Glu Gln Ser Gly Ala Met Gly Gly Glu Pro Leu Asn Thr Phe Tyr Thr  
 420 425 430  
 Gln Leu Val Leu Met Pro Gln Val Leu Gln Tyr Val Gln Tyr Val Leu  
 435 440 445  
 Leu Gly Leu Gly Gly Leu Leu Leu Leu Val Pro Val Ile Tyr Gln Leu  
 450 455 460  
 Arg Ser Gln Glu Lys Cys Phe Leu Phe Trp Ser Gly Ser Lys Lys Gly  
 465 470 475 480  
 Ser Gln Asp Lys Glu Ala Ile Gln Ala Tyr Ser Glu Ser Leu Met Ser  
 485 490 495  
 Pro Ala Ala Lys Gly Thr Val Leu Gln Glu Ala Lys Leu

500

505

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2032 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: misc. feature

(B) LOCATION: 40..1926

(D) OTHER INFORMATION: /Function = "Nucleotides 40 through  
1926 encode the amino acid sequence  
for the Drosophila Melanogaster  
Scavenger Receptor Class CI."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GACCGTATCT ATACATTAAG TTCGTAATAT CTCTGCGGAA TGGAATTTTT CTGGACTCTG	60
GCTGTGATTG TGATATATTG TATAGGTCAC ATTCATGGAC GATGTGAAAG ATCTATAGAT	120
TTGGATAATG GAAGTATAAA TTATCGACAG AGAAATATAG TGAGATTCAG ATGCAATCGC	180
GGCTACACTT TGCAGGGAAC AGTAATGCAA ACTTGCGATC GAGATGGTCG CCTTCGAGGC	240
GAAAAACCAT TCTGTGCCAG TAGGGGATGT GCGAGGCCCG AGGATCCGGA GAACGGACAC	300
GTCGAAAATC TTTCCCTAAG GCGGATGTC GTGTGCCACG ATGGCTATGT CTTGGTCGGT	360
GGTCGCACTG CCTACTGCGA TGGAGAAAGA TGGAGCACCC AGCTGGGATC GTGTGGAAGG	420
AGCAACCACA CAAGAGATCA TTCTTGCGAT TTCGAGAGCG AGGATCAGTG CGGTTGGGAG	480
GCGGAGACAA CCTTCCGACG ACCCTGGAAG CGAGTCAGCA CGGTATCCGA TATTCACTCC	540
CTAAGAACGG GACCCCGCCA CGATCACACG TTTAAAAACG AATCCGGTGG TCATTACATG	600
CGCATGGAAA CCCAAATGGG GGCTTATGGA AGCTACCATC TGCTATCGCC GATCTATCCC	660
AGATCCCTCA CCCTGAAGAC CGCCTGCTGC TTTGATTCC ACTACTTCAT GTTTGGCGCT	720
GGTGTGGATA ATCTGGTGGT GTCCGTAA AAA CCCGTTTCGA TGCCAATGGC AACCATGTGG	780
AATAGGTTCA GAGCCAATTG CAGCAAATTT GAGATATCTG GTCAGCAGGG AACCCAGTGG	840
CTAGAGCACA CGATCAGCAT TGACGAGATG CAAGAGGACT TCCAGGTGAT ATTCACGGCA	900
ACGGATGCAA GATCCCAATT CGGAGATATT GCCATCGATG ATGTAAAGCT AATGACAGGC	960
AGTGAGTGTG GCACAAACGG ATTTAGCACC ACCACAGAAC CAACGGCTCC GACAGGCAGC	1020
AACGAGCAAC CACTGGTCTA CGATATGATA AGTTGTTTCTG GTCGATGCGG AACATCAATG	1080
TCGGCCTCCA ATATAACCA CAATGGTATA GTCATGGGAT GTGGATGTAA TGACGAGTGC	1140
CTTTCGGATG AGACTTGTTG CCTAAACTAT TTGGAGGAGT GCACAAAGGA GCTGCTCACC	1200
ACGACCGAGG ATGATATTAG TTCCCTGCCC CCAACGGTCA CATCAACAAG CACAAGCACT	1260
ACGAGGAAGT CAACAACAAC AACAACCACA AGCACGACTA CTACAAGTAC AACAACAAC	1320
AAAAGGCCAA CCACAACCAC AACAACAACA AAGGCCACAA CTACAAAGCG AACAACAACC	1380
ACTAAAAAAC CGACAACAAC TTCAACAACG CCGAAGCCAA CAACAACGAC TTCAACCACA	1440
CCAAAGTCTA CAACTTCTAC AACGTCTACA ACTTCAACAA CACCAACGAC AACAAC	1500

ATAAATGTGT TTACAACAAA GAAAACAACA ATAATGATCC CTACTTCCAG TACCGAAAAG 1560  
 ACTACAGGCA TCATCACCAC CATGAAGACA CGCAAGCGCA TCACTTGGAA CGTTGATCCT 1620  
 CAGGACATCG AGGGTCACAT GGACACGAGC GGAAGTACCC CCAATCCAGC TTTAGTAGTA 1680  
 CTTTACCTGC TACTCGGCAT TGTTCTGGTG GTAGTTCTGG CCAACGTCGT TAATCGCTGG 1740  
 ATAATACCAA TCACTGGATC AAAGACCAGC AGCGAAAAGG CTGTGAGATT CAAGAAGGCA 1800  
 TTCGATAGTC TGAAGAAGCA ACGGAAAAGA AACAGCATGG ATGATCAGCC GTTATGCGAC 1860  
 TCCGATAACG ACGATGTAGA GTATTTGAA GAAATGGGCG TGGACATACG ACATAGGACC 1920  
 GATCTATGAG GGTAATCCCC AGTGATACCA AAACAAACGC TTAGGCCTGT GCCTATTGTA 1980  
 TAGGATGTTT CTAATGTGT ATGCAAGAAT CGAATAAAAG AAAATATGCA AC 2032

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 629 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (iii) HYPOTHETICAL: NO

## (v) FRAGMENT TYPE: internal

## (ix) FEATURE:

- (A) NAME/KEY: misc. feature
- (B) LOCATION: 1..629

## (D) OTHER INFORMATION: /Function = "Amino acid sequence for the Drosophila Melanogaster Scavenger Receptor Class CI."

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 30..353
- (D) OTHER INFORMATION: /note= "Positions 30-32, 90-92, 129-131, 180-182, 253-255 and 351-353 represent potential N-glycosylation sites."

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1..20
- (D) OTHER INFORMATION: /note= "Amino acids 1-20 represent a putative signal sequence."

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 21..74
- (D) OTHER INFORMATION: /note= "Amino acids 21-74 represent complement control protein domain number 1."

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 75..127
- (D) OTHER INFORMATION: /note= "Amino acids 75-127 represent complement control protein domain number 2."

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 128..312
- (D) OTHER INFORMATION: /note= "Amino acids 128-312 represent an MAM domain."

## (ix) FEATURE:

- (A) NAME/KEY: Disulfide-bond
- (B) LOCATION: 22..381
- (D) OTHER INFORMATION: /note= "The cysteines at positions 22, 45, 59, 72, 77, 99, 113, 125, 136, 144, 216, 217, 254, 310, 339, 343, 361, 363, 367, 373, 374 and 381 represent potential disulfide linkages."

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site

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(B) LOCATION: 338..381  
 (D) OTHER INFORMATION: /note= "Amino acids 338-381 represent a somatomedin B domain."

(ix) FEATURE:  
 (A) NAME/KEY: Modified-site  
 (B) LOCATION: 387..514  
 (D) OTHER INFORMATION: /note= "Amino acids 387-514 represent a mucin-like potential O-linked glycosylation region."

(ix) FEATURE:  
 (A) NAME/KEY: Domain  
 (B) LOCATION: 544..564  
 (D) OTHER INFORMATION: /note= "Amino acids 544-565 represent a putative TM domain."

(ix) FEATURE:  
 (A) NAME/KEY: Domain  
 (B) LOCATION: 565..629  
 (D) OTHER INFORMATION: /note= "Amino acids 565-629 represent a putative cytoplasmic domain."

(ix) FEATURE:  
 (A) NAME/KEY: Modified-site  
 (B) LOCATION: 576..602  
 (D) OTHER INFORMATION: /note= "Amino acids 576-579 and 599-602 represent casein kinase II sites."

(ix) FEATURE:  
 (A) NAME/KEY: Modified-site  
 (B) LOCATION: 578..592  
 (D) OTHER INFORMATION: /note= "Amino acids 578-580 and 590-592 represent protein kinase C sites."

(ix) FEATURE:  
 (A) NAME/KEY: Modified-site  
 (B) LOCATION: 596..599  
 (D) OTHER INFORMATION: /note= "Amino acids 596-599 represent a cAMP protein kinase site."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met	Glu	Phe	Phe	Trp	Thr	Leu	Ala	Val	Ile	Val	Ile	Tyr	Cys	Ile	Gly	1	5	10	15
His	Ile	His	Gly	Arg	Cys	Glu	Arg	Ser	Ile	Asp	Leu	Asp	Asn	Gly	Ser	20	25	30	
Ile	Asn	Tyr	Arg	Gln	Arg	Asn	Ile	Val	Arg	Phe	Arg	Cys	Asn	Arg	Gly	35	40	45	
Tyr	Thr	Leu	Gln	Gly	Thr	Val	Met	Gln	Thr	Cys	Asp	Arg	Asp	Gly	Arg	50	55	60	
Leu	Arg	Gly	Glu	Lys	Pro	Phe	Cys	Ala	Ser	Arg	Gly	Cys	Ala	Arg	Pro	65	70	75	80
Glu	Asp	Pro	Glu	Asn	Gly	His	Val	Glu	Asn	Leu	Ser	Leu	Arg	Ala	Asp	85	90	95	
Val	Val	Cys	His	Asp	Gly	Tyr	Val	Leu	Val	Gly	Gly	Arg	Thr	Ala	Tyr	100	105	110	
Cys	Asp	Gly	Glu	Arg	Trp	Ser	Thr	Gln	Leu	Gly	Ser	Cys	Arg	Arg	Ser	115	120	125	
Asn	His	Thr	Arg	Asp	His	Ser	Cys	Asp	Phe	Glu	Ser	Glu	Asp	Gln	Cys	130	135	140	
Gly	Trp	Glu	Ala	Glu	Thr	Thr	Phe	Arg	Arg	Pro	Trp	Lys	Arg	Val	Ser	145	150	155	160
Thr	Val	Ser	Asp	Ile	His	Ser	Leu	Arg	Thr	Gly	Pro	Arg	His	Asp	His				

165										170					175				
Thr	Phe	Lys	Asn	Glu	Ser	Gly	Gly	His	Tyr	Met	Arg	Met	Glu	Thr	Gln				
			180					185					190						
Met	Gly	Ala	Tyr	Gly	Ser	Tyr	His	Leu	Leu	Ser	Pro	Ile	Tyr	Pro	Arg				
		195					200					205							
Ser	Leu	Thr	Leu	Lys	Thr	Ala	Cys	Cys	Phe	Arg	Phe	His	Tyr	Phe	Met				
	210					215					220								
Phe	Gly	Ala	Gly	Val	Asp	Asn	Leu	Val	Val	Ser	Val	Lys	Pro	Val	Ser				
225					230					235					240				
Met	Pro	Met	Ala	Thr	Met	Trp	Asn	Arg	Phe	Arg	Ala	Asn	Cys	Ser	Lys				
				245					250					255					
Phe	Glu	Ile	Ser	Gly	Gln	Gln	Gly	Thr	Gln	Trp	Leu	Glu	His	Thr	Ile				
			260					265					270						
Ser	Ile	Asp	Glu	Met	Gln	Glu	Asp	Phe	Gln	Val	Ile	Phe	Thr	Ala	Thr				
		275					280					285							
Asp	Ala	Arg	Ser	Gln	Phe	Gly	Asp	Ile	Ala	Ile	Asp	Asp	Val	Lys	Leu				
	290					295					300								
Met	Thr	Gly	Ser	Glu	Cys	Gly	Thr	Asn	Gly	Phe	Ser	Thr	Thr	Thr	Glu				
305					310					315					320				
Pro	Thr	Ala	Pro	Thr	Gly	Ser	Asn	Glu	Gln	Pro	Leu	Val	Tyr	Asp	Met				
				325					330					335					
Ile	Ser	Cys	Ser	Gly	Arg	Cys	Gly	Thr	Ser	Met	Ser	Ala	Ser	Asn	Ile				
			340					345					350						
Thr	Asn	Asn	Gly	Ile	Val	Met	Gly	Cys	Gly	Cys	Asn	Asp	Glu	Cys	Leu				
		355					360					365							
Ser	Asp	Glu	Thr	Cys	Cys	Leu	Asn	Tyr	Leu	Glu	Glu	Cys	Thr	Lys	Glu				
	370					375					380								
Leu	Leu	Thr	Thr	Thr	Glu	Asp	Asp	Ile	Ser	Ser	Leu	Pro	Pro	Thr	Val				
385					390					395					400				
Thr	Ser	Thr	Ser	Thr	Ser	Thr	Thr	Arg	Lys	Ser	Thr	Thr	Thr	Thr	Thr				
				405					410					415					
Thr	Ser	Thr	Thr	Thr	Thr	Ser	Thr	Thr	Thr	Thr	Lys	Arg	Pro	Thr	Thr				
			420					425					430						
Thr	Thr	Thr	Thr	Thr	Lys	Ala	Thr	Thr	Thr	Lys	Arg	Thr	Thr	Thr	Thr				
			435				440					445							
Lys	Lys	Pro	Thr	Thr	Thr	Ser	Thr	Thr	Pro	Lys	Pro	Thr	Thr	Thr	Thr				
	450					455					460								
Ser	Thr	Thr	Pro	Lys	Ser	Thr	Thr	Ser	Thr	Thr	Ser	Thr	Thr	Ser	Thr				
465					470					475					480				
Thr	Pro	Thr	Thr	Thr	Thr	Thr	Ile	Asn	Val	Phe	Thr	Thr	Lys	Lys	Thr				
				485				490					495						
Thr	Ile	Met	Ile	Pro	Thr	Ser	Ser	Thr	Glu	Lys	Thr	Thr	Gly	Ile	Ile				
			500					505					510						
Thr	Thr	Met	Lys	Thr	Arg	Lys	Arg	Ile	Thr	Trp	Asn	Val	Asp	Pro	Gln				
		515					520					525							



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Asp Ile Glu Gly His Met Asp Thr Ser Gly Ser Thr Pro Asn Pro Ala  
 530 535 540  
 Leu Val Val Leu Tyr Leu Leu Leu Gly Ile Val Leu Val Val Val Leu  
 545 550 555 560  
 Ala Asn Val Val Asn Arg Trp Ile Ile Pro Ile Thr Gly Ser Lys Thr  
 565 570 575  
 Ser Ser Glu Lys Ala Val Arg Phe Lys Lys Ala Phe Asp Ser Leu Lys  
 580 585 590  
 Lys Gln Arg Lys Arg Asn Ser Met Asp Asp Gln Pro Leu Cys Asp Ser  
 595 600 605  
 Asp Asn Asp Asp Val Glu Tyr Phe Glu Glu Met Gly Val Asp Ile Arg  
 610 615 620  
 His Arg Thr Asp Leu  
 625

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1785 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (iii) HYPOTHETICAL: NO

## (iv) ANTI-SENSE: NO

## (ix) FEATURE:

## (A) NAME/KEY: CDS

## (B) LOCATION: 51..1577

 (D) OTHER INFORMATION: /Function = "Nucleotides 51 through  
 1577 encode the amino acid sequence  
 for the murine Scavenger Receptor  
 Class BI."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCGTCTCCTT CAGGTCCTGA GCCCGAGAG CCCCTTCCGC GCACGCGGAC ATG GGC	56
Met Gly	
1	
GGC AGC TCC AGG GCG CGC TGG GTG GCC TTG GGG TTG GGC GCC CTG GGG	104
Gly Ser Ser Arg Ala Arg Trp Val Ala Leu Gly Leu Gly Ala Leu Gly	
5 10 15	
CTG CTG TTT GCT GCG CTC GGC GTT GTC ATG ATC CTC ATG GTG CCC TCC	152
Leu Leu Phe Ala Ala Leu Gly Val Val Met Ile Leu Met Val Pro Ser	
20 25 30	
CTC ATC AAG CAG CAG GTG CTC AAG AAT GTC CGC ATA GAC CCG AGC AGC	200
Leu Ile Lys Gln Gln Val Leu Lys Asn Val Arg Ile Asp Pro Ser Ser	
35 40 45 50	
CTG TCC TTC GGG ATG TGG AAG GAG ATC CCC GTC CCT TTC TAC TTG TCT	248
Leu Ser Phe Gly Met Trp Lys Glu Ile Pro Val Pro Phe Tyr Leu Ser	
55 60 65	
GTC TAC TTC TTC GAA GTG GTC AAC CCA AAC GAG GTC CTC AAC GGC CAG	296
Val Tyr Phe Phe Glu Val Val Asn Pro Asn Glu Val Leu Asn Gly Gln	
70 75 80	
AAG CCA GTA GTC CGG GAG CGT GGA CCC TAT GTC TAC AGG GAG TTC AGA	344
Lys Pro Val Val Arg Glu Arg Gly Pro Tyr Val Tyr Arg Glu Phe Arg	
85 90 95	
CAA AAG GTC AAC ATC ACC TTC AAT GAC AAC GAC ACC GTG TCC TTC GTG	392
Gln Lys Val Asn Ile Thr Phe Asn Asp Asn Asp Thr Val Ser Phe Val	
100 105 110	

GAG Glu 115	AAC Asn	CGC Arg	AGC Ser	CTC Leu	CAT His	TTC Phe	CAG Gln	CCT Pro	GAC Asp	AAG Lys	TCG Ser	CAT His	GGC Gly	TCA Ser	GAG Glu	440
							120				125				130	
AGT Ser	GAC Asp	TAC Tyr	ATT Ile	GTA Val	CTG Leu	CCT Pro	AAC Asn	ATC Ile	TTG Leu	GTC Val	CTG Leu	GGG Gly	GGC Gly	TCG Ser	ATA Ile	488
					135				140						145	
TTG Leu	ATG Met	GAG Glu	AGC Ser	AAG Lys	CCT Pro	GTG Val	AGC Ser	CTG Leu	AAG Lys	CTG Leu	ATG Met	ATG Met	ACC Thr	TTG Leu	GCG Ala	536
					150				155						160	
CTG Leu	GTC Val	ACC Thr	ATG Met	GGC Gly	CAG Gln	CGT Arg	GCT Ala	TTT Phe	ATG Met	AAC Asn	CGC Arg	ACA Thr	GTT Val	GGT Gly	GAG Glu	584
					165			170							175	
ATC Ile	CTG Leu	TGG Trp	GGC Gly	TAT Tyr	GAC Asp	GAT Asp	CCC Pro	TTC Phe	GTG Val	CAT His	TTT Phe	CTC Leu	AAC Asn	ACG Thr	TAC Tyr	632
		180				185					190					
CTC Leu	CCA Pro	GAC Asp	ATG Met	CTT Leu	CCC Pro	ATA Ile	AAG Lys	GGC Gly	AAA Lys	TTT Phe	GGC Gly	CTG Leu	TTT Phe	GTT Val	GGG Gly	680
					200					205					210	
ATG Met	AAC Asn	AAC Asn	TCG Ser	AAT Asn	TCT Ser	GGG Gly	GTC Val	TTC Phe	ACT Thr	GTC Val	TTC Phe	ACG Thr	GGC Gly	GTC Val	CAG Gln	728
					215				220						225	
AAT Asn	TTC Phe	AGC Ser	AGG Arg	ATC Ile	CAT His	CTG Leu	GTG Val	GAC Asp	AAA Lys	TGG Trp	AAC Asn	GGA Gly	CTC Leu	AGC Ser	AAG Lys	776
					230				235					240		
ATC Ile	GAT Asp	TAT Tyr	TGG Trp	CAT His	TCA Ser	GAG Glu	CAG Gln	TGT Cys	AAC Asn	ATG Met	ATC Ile	AAT Asn	GGG Gly	ACT Thr	TCC Ser	824
			245				250					255				
GGG Gly	CAG Gln	ATG Met	TGG Trp	GCA Ala	CCC Pro	TTC Phe	ATG Met	ACA Thr	CCC Pro	GAA Glu	TCC Ser	TCG Ser	CTG Leu	GAA Glu	TTC Phe	872
			260				265					270				
TTC Phe	AGC Ser	CCG Pro	GAG Glu	GCA Ala	TGC Cys	AGG Arg	TCC Ser	ATG Met	AAG Lys	CTG Leu	ACC Thr	TAC Tyr	AAC Asn	GAA Glu	TCA Ser	920
					280					285					290	
AGG Arg	GTG Val	TTT Phe	GAA Glu	GGC Gly	ATT Ile	CCC Pro	ACG Thr	TAT Tyr	CGC Arg	TTC Phe	ACG Thr	GCC Ala	CCC Pro	GAT Asp	ACT Thr	968
					295				300					305		
CTG Leu	TTT Phe	GCC Ala	AAC Asn	GGG Gly	TCC Ser	GTG Val	TAC Tyr	CCA Pro	CCC Pro	AAC Asn	GAA Glu	GGC Gly	TTC Phe	TGC Cys	CCA Pro	1016
					310			315						320		
TGC Cys	CGA Arg	GAG Glu	TCT Ser	GGC Gly	ATT Ile	CAG Gln	AAT Asn	GTG Val	AGC Ser	ACC Thr	TGC Cys	AGG Arg	TTT Phe	GGT Gly	GCG Ala	1064
			325				330					335				
CCT Pro	CTG Leu	TTT Phe	CTC Leu	TCC Ser	CAC His	CCC Pro	CAC His	TTT Phe	TAC Tyr	AAC Asn	GCC Ala	GAC Asp	CCT Pro	GTG Val	TTG Leu	1112
					340		345				350					
TCA Ser	GAA Glu	GCT Ala	GTT Val	CTT Leu	GGT Gly	CTG Leu	AAC Asn	CCT Pro	AAC Asn	CCA Pro	AAG Lys	GAG Glu	CAT His	TCC Ser	TTG Leu	1160
					360					365					370	
TTC Phe	CTA Leu	GAC Asp	ATC Ile	CAT His	CCG Pro	GTG Val	ACT Thr	GGG Gly	ATC Ile	CCC Pro	ATG Met	AAC Asn	TGT Cys	TCT Ser	GTG Val	1208
					375				380						385	
AAG Lys	ATG Met	CAG Gln	CTG Leu	AGC Ser	CTC Leu	TAC Tyr	ATC Ile	AAA Lys	TCT Ser	GTG Val	AAG Lys	GGC Gly	ATC Ile	GGG Gly	CAA Gln	1256

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390	395	400	
ACA GGG AAG ATC GAG CCA GTA GTT CTG CCG TTG CTG TGG TTC GAA CAG			1304
Thr Gly Lys Ile Glu Pro Val Val Leu Pro Leu Leu Trp Phe Glu Gln			
405	410	415	
AGC GGA GCA ATG GGT GGC AAG CCC CTG AGC ACG TTC TAC ACG CAG CTG			1352
Ser Gly Ala Met Gly Gly Lys Pro Leu Ser Thr Phe Tyr Thr Gln Leu			
420	425	430	
GTG CTG ATG CCC CAG GTT CTT CAC TAC GCG CAG TAT GTG CTG CTG GGG			1400
Val Leu Met Pro Gln Val Leu His Tyr Ala Gln Tyr Val Leu Leu Gly			
435	440	445	450
CTT GGA GGC CTC CTG TTG CTG GTG CCC ATC ATC TGC CAA CTG CGC AGC			1448
Leu Gly Gly Leu Leu Leu Val Pro Ile Ile Cys Gln Leu Arg Ser			
455	460	465	
CAG GAG AAA TGC TTT TTG TTT TGG AGT GGT AGT AAA AAG GGC TCC CAG			1496
Gln Glu Lys Cys Phe Leu Phe Trp Ser Gly Ser Lys Lys Gly Ser Gln			
470	475	480	
GAT AAG GAG GCC ATT CAG GCC TAC TCT GAG TCC CTG ATG TCA CCA GCT			1544
Asp Lys Glu Ala Ile Gln Ala Tyr Ser Glu Ser Leu Met Ser Pro Ala			
485	490	495	
GCC AAG GGC ACG GTG CTG CAA GAA GCC AAG CTA TAGGGTCCTG AAGACACTAT			1597
Ala Lys Gly Thr Val Leu Gln Glu Ala Lys Leu			
500	505		
AAGCCCCCA AACCTGATAG CTTGGTCAGA CCAGCCACCC AGTCCCTACA CCCCCTTCT			1657
TGAGGACTCT CTCAGCGGAC AGCCCACCAG TGCCATGGCC TGAGCCCCCA GATGTCACAC			1717
CTGTCCGCAC GCACGGCACA TGGATGCCCA CGCATGTGCA AAAACAAC TC AGGGACCAGG			1777
GACAGACC			1785

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 509 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (ix) FEATURE:

- (A) NAME/KEY: misc. feature
- (B) LOCATION: 1..509
- (D) OTHER INFORMATION: /Function = "Amino acid sequence for

the murine Scavenger Receptor Class BI."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Gly Gly Ser Ser Arg Ala Arg Trp Val Ala Leu Gly Leu Gly Ala		
1	5	10
Leu Gly Leu Leu Phe Ala Ala Leu Gly Val Val Met Ile Leu Met Val		
20	25	30
Pro Ser Leu Ile Lys Gln Gln Val Leu Lys Asn Val Arg Ile Asp Pro		
35	40	45
Ser Ser Leu Ser Phe Gly Met Trp Lys Glu Ile Pro Val Pro Phe Tyr		
50	55	60
Leu Ser Val Tyr Phe Phe Glu Val Val Asn Pro Asn Glu Val Leu Asn		
65	70	75
Gly Gln Lys Pro Val Val Arg Glu Arg Gly Pro Tyr Val Tyr Arg Glu		
85	90	95
Phe Arg Gln Lys Val Asn Ile Thr Phe Asn Asp Asn Asp Thr Val Ser		

100					105					110					
Phe	Val	Glu	Asn	Arg	Ser	Leu	His	Phe	Gln	Pro	Asp	Lys	Ser	His	Gly
		115					120					125			
Ser	Glu	Ser	Asp	Tyr	Ile	Val	Leu	Pro	Asn	Ile	Leu	Val	Leu	Gly	Gly
	130					135					140				
Ser	Ile	Leu	Met	Glu	Ser	Lys	Pro	Val	Ser	Leu	Lys	Leu	Met	Met	Thr
	145					150					155				160
Leu	Ala	Leu	Val	Thr	Met	Gly	Gln	Arg	Ala	Phe	Met	Asn	Arg	Thr	Val
				165					170					175	
Gly	Glu	Ile	Leu	Trp	Gly	Tyr	Asp	Asp	Pro	Phe	Val	His	Phe	Leu	Asn
			180					185					190		
Thr	Tyr	Leu	Pro	Asp	Met	Leu	Pro	Ile	Lys	Gly	Lys	Phe	Gly	Leu	Phe
		195					200					205			
Val	Gly	Met	Asn	Asn	Ser	Asn	Ser	Gly	Val	Phe	Thr	Val	Phe	Thr	Gly
	210					215					220				
Val	Gln	Asn	Phe	Ser	Arg	Ile	His	Leu	Val	Asp	Lys	Trp	Asn	Gly	Leu
	225					230					235				240
Ser	Lys	Ile	Asp	Tyr	Trp	His	Ser	Glu	Gln	Cys	Asn	Met	Ile	Asn	Gly
			245						250					255	
Thr	Ser	Gly	Gln	Met	Trp	Ala	Pro	Phe	Met	Thr	Pro	Glu	Ser	Ser	Leu
			260					265					270		
Glu	Phe	Phe	Ser	Pro	Glu	Ala	Cys	Arg	Ser	Met	Lys	Leu	Thr	Tyr	Asn
		275					280					285			
Glu	Ser	Arg	Val	Phe	Glu	Gly	Ile	Pro	Thr	Tyr	Arg	Phe	Thr	Ala	Pro
	290					295					300				
Asp	Thr	Leu	Phe	Ala	Asn	Gly	Ser	Val	Tyr	Pro	Pro	Asn	Glu	Gly	Phe
	305					310					315				320
Cys	Pro	Cys	Arg	Glu	Ser	Gly	Ile	Gln	Asn	Val	Ser	Thr	Cys	Arg	Phe
			325						330					335	
Gly	Ala	Pro	Leu	Phe	Leu	Ser	His	Pro	His	Phe	Tyr	Asn	Ala	Asp	Pro
			340					345					350		
Val	Leu	Ser	Glu	Ala	Val	Leu	Gly	Leu	Asn	Pro	Asn	Pro	Lys	Glu	His
		355					360						365		
Ser	Leu	Phe	Leu	Asp	Ile	His	Pro	Val	Thr	Gly	Ile	Pro	Met	Asn	Cys
	370					375					380				
Ser	Val	Lys	Met	Gln	Leu	Ser	Leu	Tyr	Ile	Lys	Ser	Val	Lys	Gly	Ile
	385					390					395				400
Gly	Gln	Thr	Gly	Lys	Ile	Glu	Pro	Val	Val	Leu	Pro	Leu	Leu	Trp	Phe
			405						410					415	
Glu	Gln	Ser	Gly	Ala	Met	Gly	Gly	Lys	Pro	Leu	Ser	Thr	Phe	Tyr	Thr
			420					425					430		
Gln	Leu	Val	Leu	Met	Pro	Gln	Val	Leu	His	Tyr	Ala	Gln	Tyr	Val	Leu
		435					440					445			
Leu	Gly	Leu	Gly	Gly	Leu	Leu	Leu	Leu	Val	Pro	Ile	Ile	Cys	Gln	Leu
	450					455					460				
Arg	Ser	Gln	Glu	Lys	Cys	Phe	Leu	Phe	Trp	Ser	Gly	Ser	Lys	Lys	Gly
	465					470					475				480

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Ser Gln Asp Lys Glu Ala Ile Gln Ala Tyr Ser Glu Ser Leu Met Ser  
485 490 495

Pro Ala Ala Lys Gly Thr Val Leu Gln Glu Ala Lys Leu  
500 505

We claim:

1. An isolated scavenger receptor protein type BI which selectively binds to low density lipoprotein and to modified lipoprotein having the characteristics of acetylated low density lipoprotein.

2. The protein of claim 1 expressed in cells selected from the group consisting of adipocytes, lung and liver.

3. The protein of claim 1 encoded by a sequence hybridizing under stringent conditions to Sequence ID No. 3.

4. The protein of claim 3 wherein the sequence is Sequence ID No. 3 or a degenerate variant thereof.

5. The protein of claim 1 having an amino acid sequence consisting essentially of the sequence shown in Sequence ID No. 4.

6. The protein of claim 1 immobilized to an inert substrate in a form useful for binding of low density lipoprotein.

7. The protein of claim 1 of human origin.

8. The protein of claim 1 expressed on the surface of a cell genetically engineered to express the protein.

9. An antibody to scavenger receptor protein which selectively binds to low density lipoprotein and to modified lipoprotein having the characteristics of acetylated low density lipoprotein.

10. The antibody of claim 9 further comprising a detectable label.

11. An isolated nucleic acid sequence comprising at least fourteen nucleotides encoding at least in part or regulating the expression of a scavenger receptor protein type BI which selectively binds to low density lipoprotein and to modified lipoprotein having the characteristics of acetylated low density lipoprotein.

12. The sequence of claim 11 expressed in cells selected from the group consisting of adipocytes, lung and liver.

13. The sequence of claim 11 hybridizing under stringent conditions to Sequence ID No. 3.

14. The sequence of claim 13 wherein the sequence is Sequence ID No. 3 or a degenerate variant thereof.

15. The sequence of claim 11 encoding an amino acid sequence consisting essentially of the sequence shown in Sequence ID No. 4.

16. The sequence of claim 11 regulating expression of genomic DNA encoding the scavenger receptor protein.

17. The sequence of claim 11 encoding the scavenger receptor protein.

18. The sequence of claim 11 which is genomic DNA.

19. The sequence of claim 11 which encodes the human scavenger receptor.

20. The sequence of claim 11 labeled with a detectable label.

21. The sequence of claim 11 encoding the scavenger receptor protein further comprising an expression vector.

22. The sequence of claim 21 further comprising a host cell suitable for expression of the scavenger receptor.

23. An isolated scavenger receptor protein type CI which selectively binds to modified lipoprotein having the characteristics of acetylated low density lipoprotein and having the structure of the scavenger receptor protein defined by Sequence ID No. 6.

24. The protein of claim 23 expressed in *Drosophila melanogaster* cells.

25. The protein of claim 23 encoded by a sequence hybridizing under stringent conditions to Sequence ID No. 5.

26. The protein of claim 25 wherein the sequence is Sequence ID No. 5 or a degenerate variant thereof.

27. The protein of claim 23 having an amino acid sequence consisting essentially of the sequence shown in Sequence ID No. 6.

28. The protein of claim 23 of human origin.

29. The protein of claim 23 expressed on the surface of a cell genetically engineered to express the protein.

30. An antibody to scavenger receptor protein which selectively binds to modified lipoprotein having the characteristics of acetylated low density lipoprotein and having the structure of the scavenger receptor protein defined by Sequence ID No. 6.

31. The antibody of claim 30 further comprising a detectable label.

32. An isolated nucleic acid sequence comprising at least fourteen nucleotides encoding at least in part or regulating the expression of a scavenger receptor protein type CI which selectively binds to modified lipoprotein having the characteristics of acetylated low density lipoprotein and having the structure of the scavenger receptor protein defined by Sequence ID No. 6.

33. The sequence of claim 32 expressed in cells of *Drosophila melanogaster*.

34. The sequence of claim 32 hybridizing under stringent conditions to Sequence ID No. 5.

35. The sequence of claim 34 wherein the sequence is Sequence ID No. 5 or a degenerate variant thereof.

36. The sequence of claim 33 encoding an amino acid sequence consisting essentially of the sequence shown in Sequence ID No. 6.

37. The sequence of claim 33 regulating expression of genomic DNA encoding the scavenger receptor protein.

38. The sequence of claim 33 encoding the scavenger receptor protein.

39. The sequence of claim 33 which is genomic DNA.

40. The sequence of claim 33 which encodes the human scavenger receptor.

41. The sequence of claim 33 labeled with a detectable label.

42. The sequence of claim 33 encoding the scavenger receptor protein further comprising an expression vector.

43. The sequence of claim 42 further comprising a host cell suitable for expression of the scavenger receptor.



44. A method for screening for a compound which alters the binding of a scavenger receptor protein selected from the group consisting of scavenger receptor protein type BI which selectively binds to low density lipoprotein and to modified lipoprotein having the characteristics of acetylated low density lipoprotein and scavenger receptor protein type CI which selectively binds to modified lipoprotein having the characteristics of acetylated low density lipoprotein and having the structure of the scavenger receptor protein defined by Sequence ID No. 6 comprising

providing an assay for binding of low density lipoprotein or modified low density lipoprotein to the scavenger receptor protein,

adding the compound to be tested to the assay, and

determining if the amount of modified low density lipoprotein or low density lipoprotein which is bound to the scavenger receptor protein is altered as compared to binding in the absence of the compound to be tested.

45. The assay of claim 44 wherein the assay includes a cell expressing the scavenger receptor protein and the compound is a nucleic acid sequence which alters expression of the scavenger receptor protein.

46. The assay of claim 44 wherein the compound is selected from a library of naturally occurring or synthetic compounds which are randomly tested for alteration of binding.

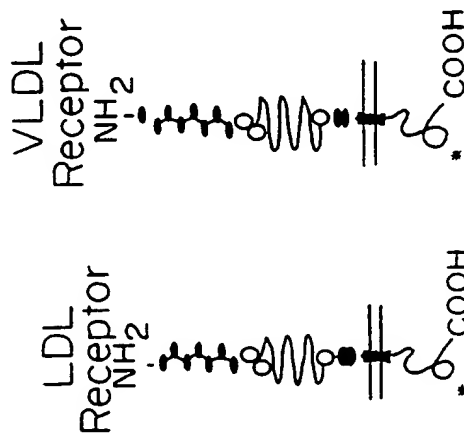
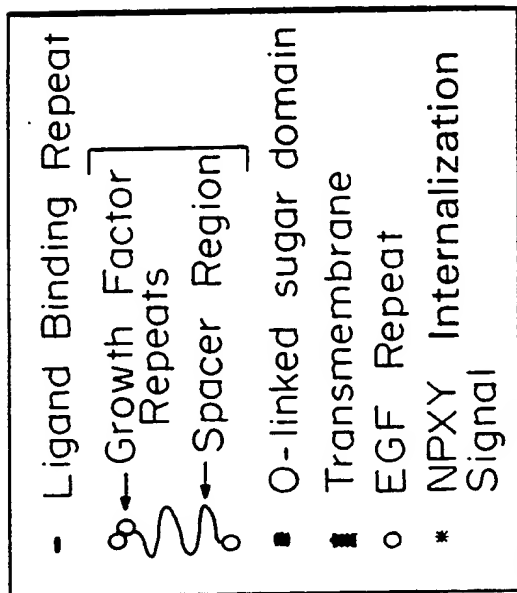
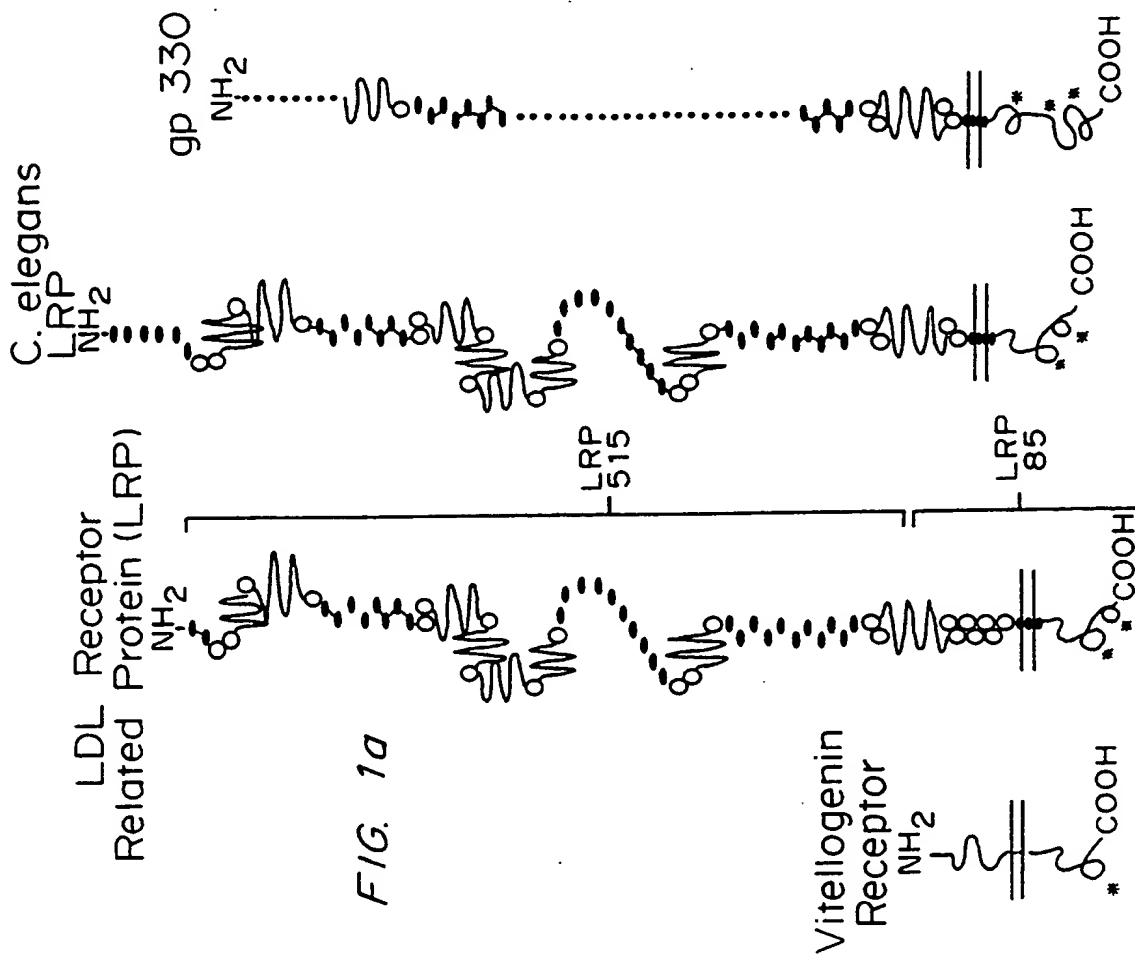
47. The assay of claim 44 wherein the compound competitively inhibits binding to the scavenger receptor protein.

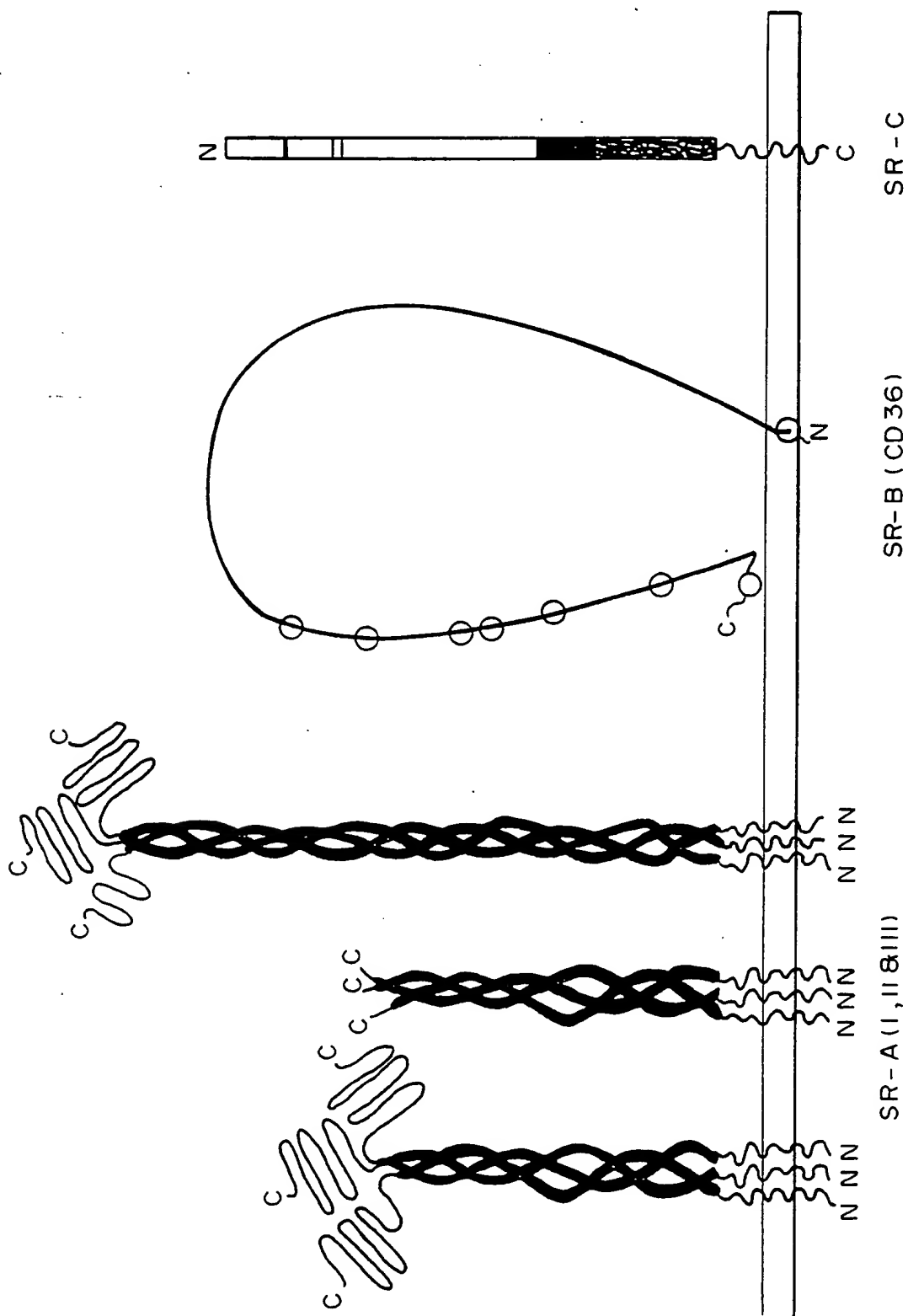
48. A method for removing low density lipoprotein from patient blood comprising reacting the blood with immobilized scavenger receptor protein type BI which selectively binds to low density lipoprotein and to modified lipoprotein having the characteristics of acetylated low density lipoprotein under conditions wherein the low density lipoprotein is bound to the scavenger receptor.

49. A method for inhibiting uptake of lipoprotein or lipids by adipocytes comprising selectively inhibiting binding of lipoprotein to the scavenger receptor protein type BI which selectively binds to low density lipoprotein and to modified lipoprotein having the characteristics of acetylated low density lipoprotein under conditions wherein the low density lipoprotein is bound to the scavenger receptor.

50. A method for screening patients for abnormal scavenger receptor protein activity or function comprising

determining the presence of a scavenger receptor protein selected from the group consisting of scavenger receptor protein type BI which selectively binds to low density lipoprotein and to modified lipoprotein having the characteristics of acetylated low density lipoprotein and scavenger receptor protein type CI which selectively binds to modified lipoprotein having the characteristics of acetylated low density lipoprotein and having the structure of the scavenger receptor protein defined by Sequence ID No. 6 in a patient sample, and comparing the scavenger receptor for to determine if the quantity present or the function of the receptor is equivalent to that present in normal cells.





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FIG. 2a

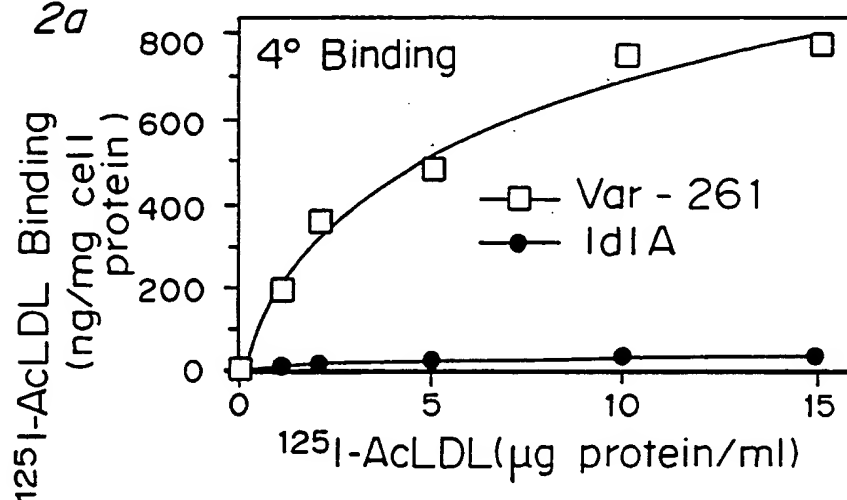


FIG. 2b

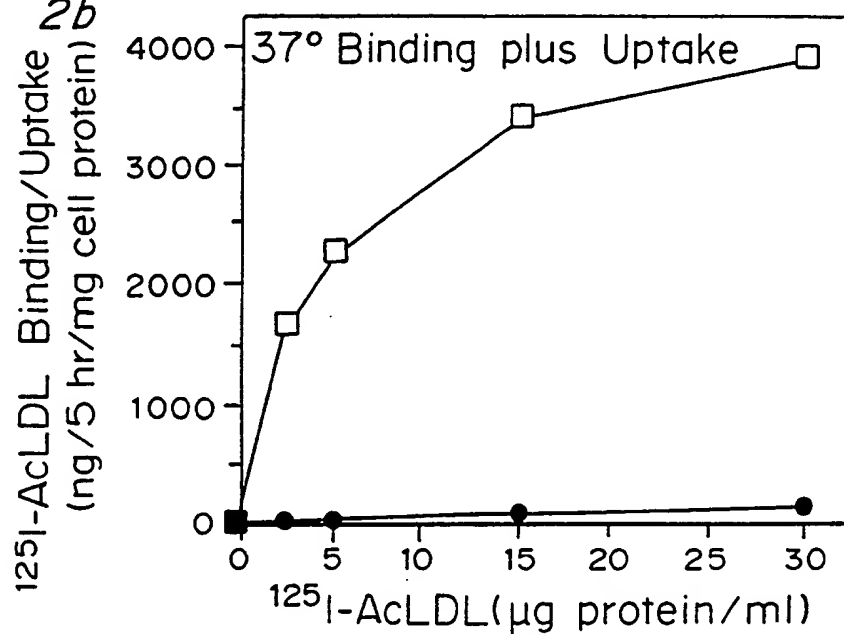
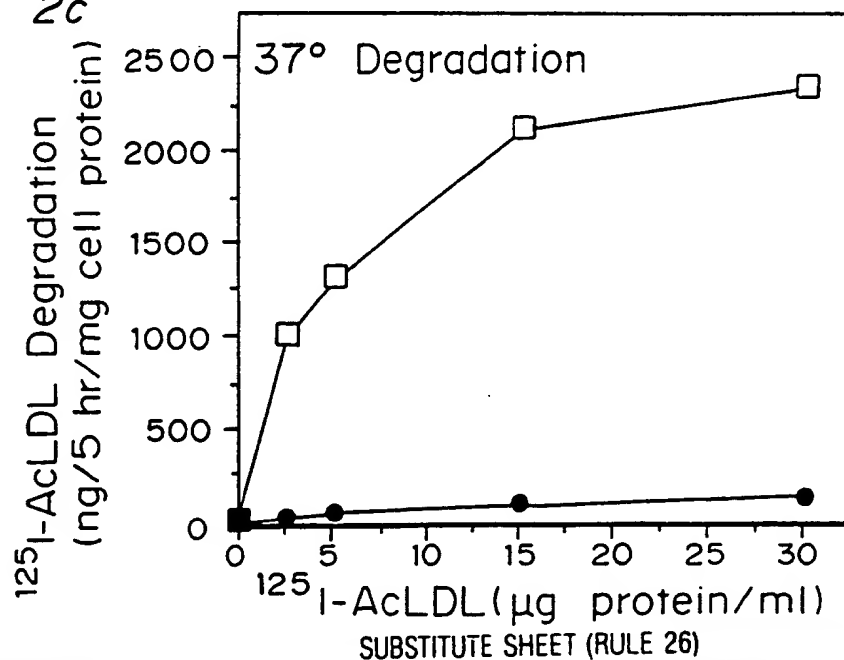
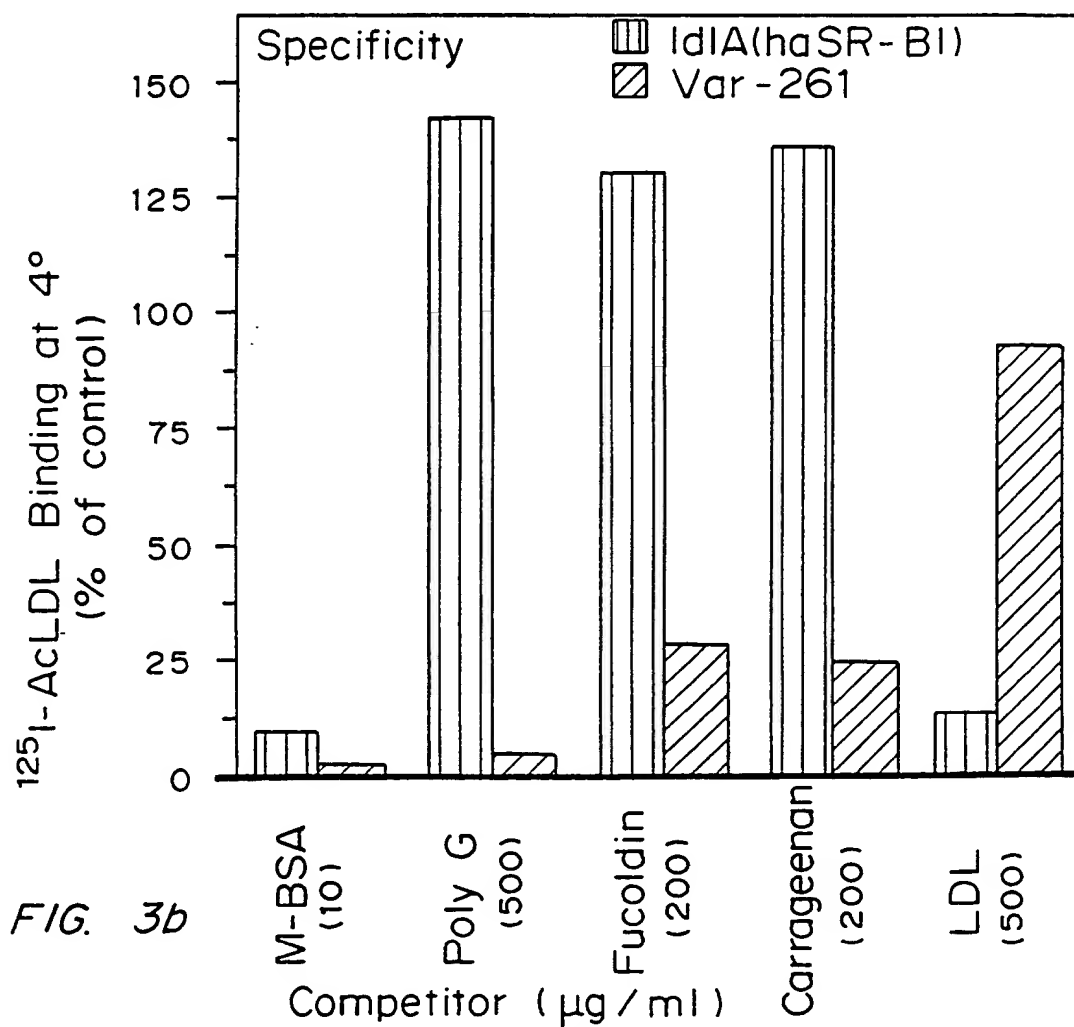
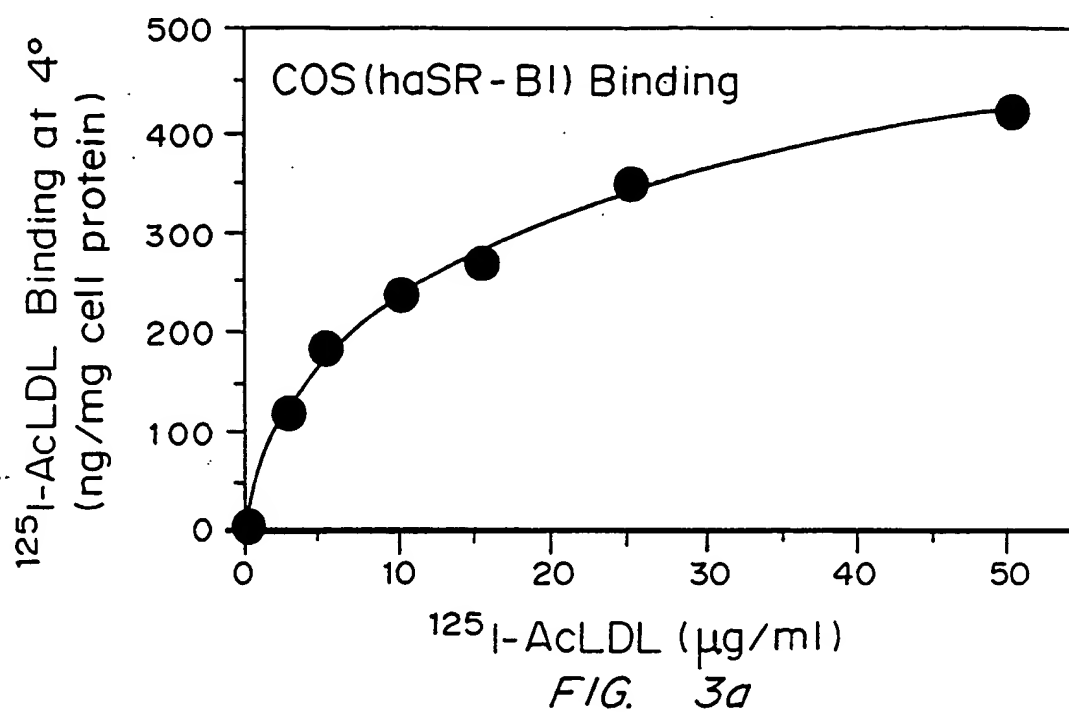


FIG. 2c



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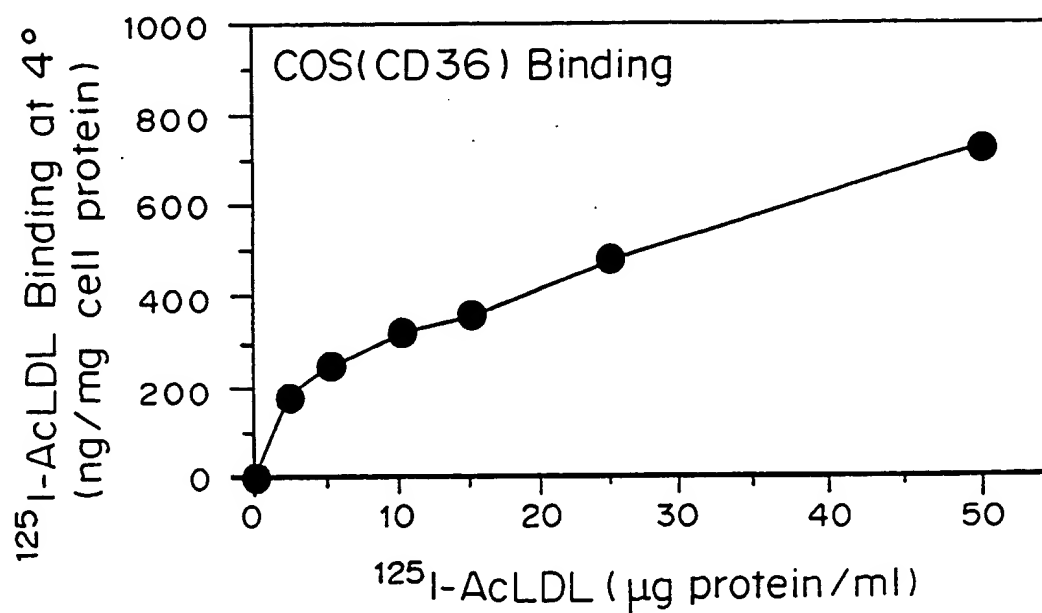


FIG. 4a

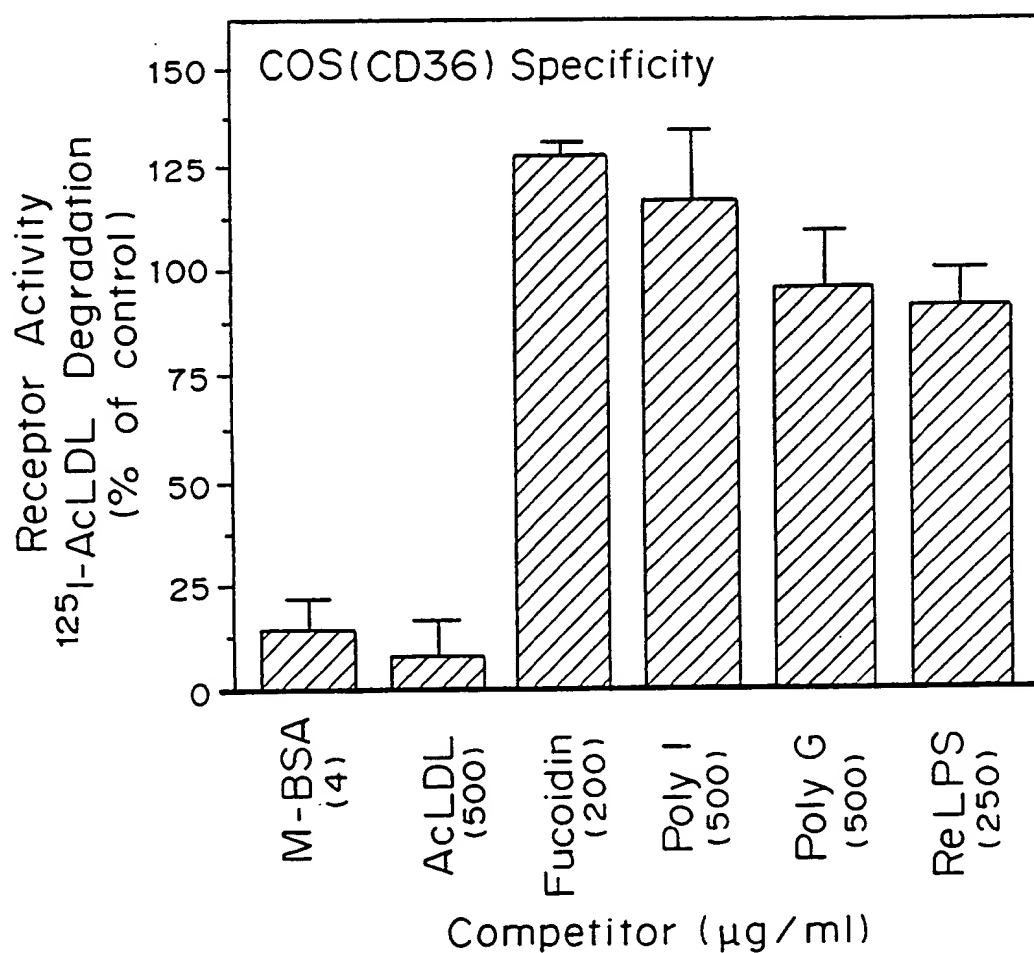


FIG. 4b

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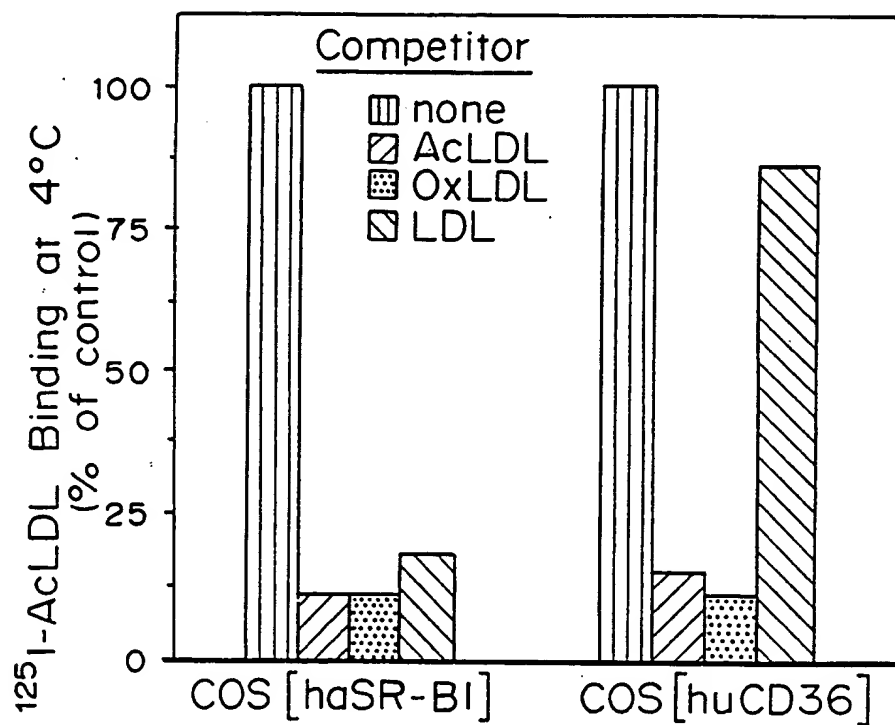


FIG. 5

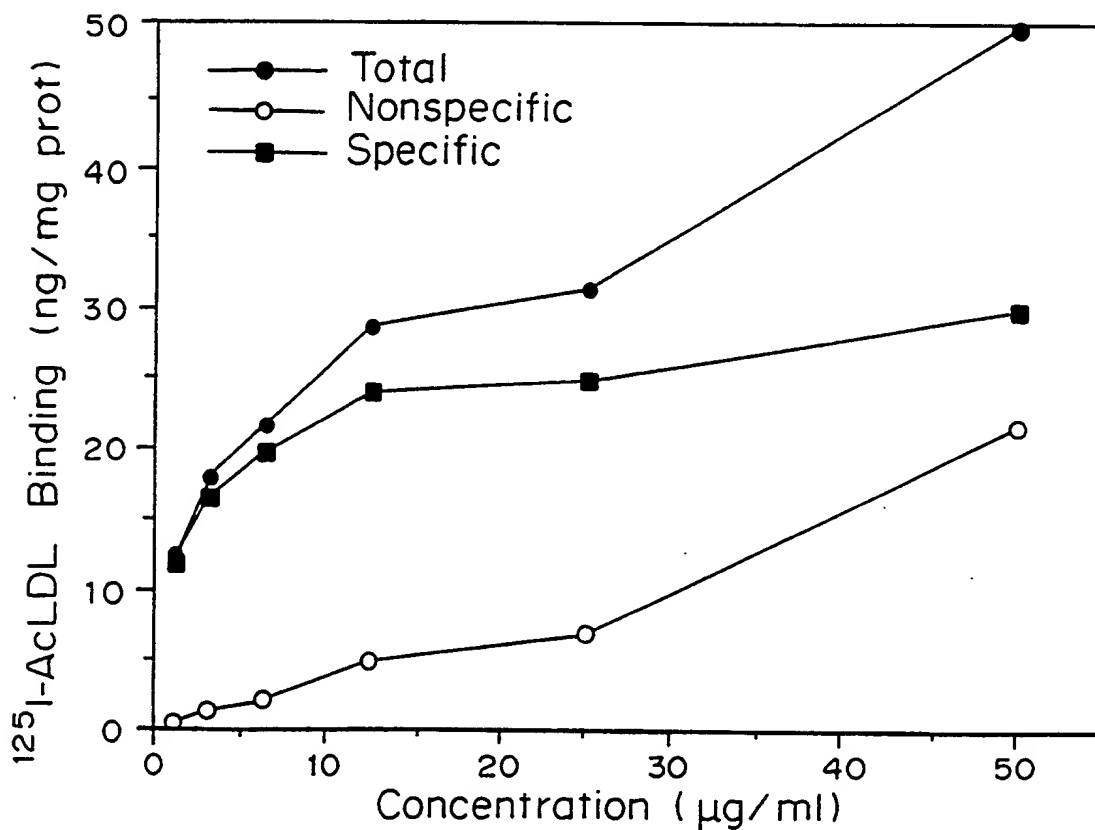


FIG. 6a

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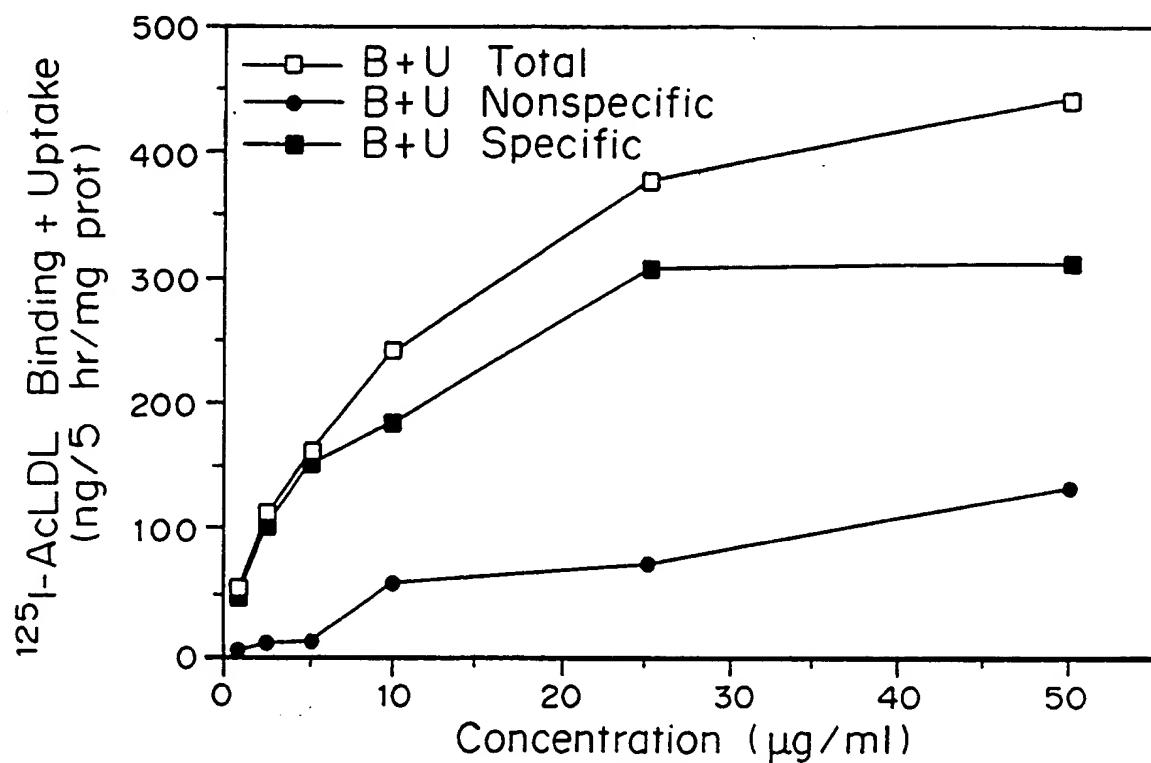


FIG. 6b

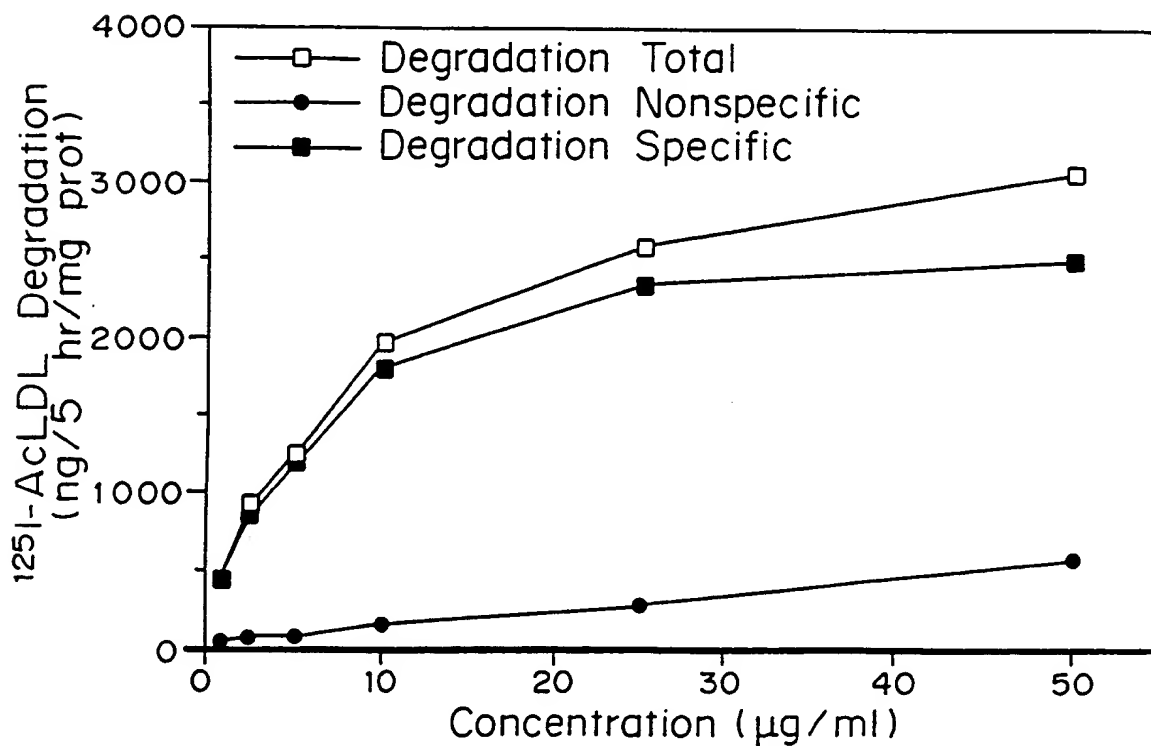


FIG. 6c

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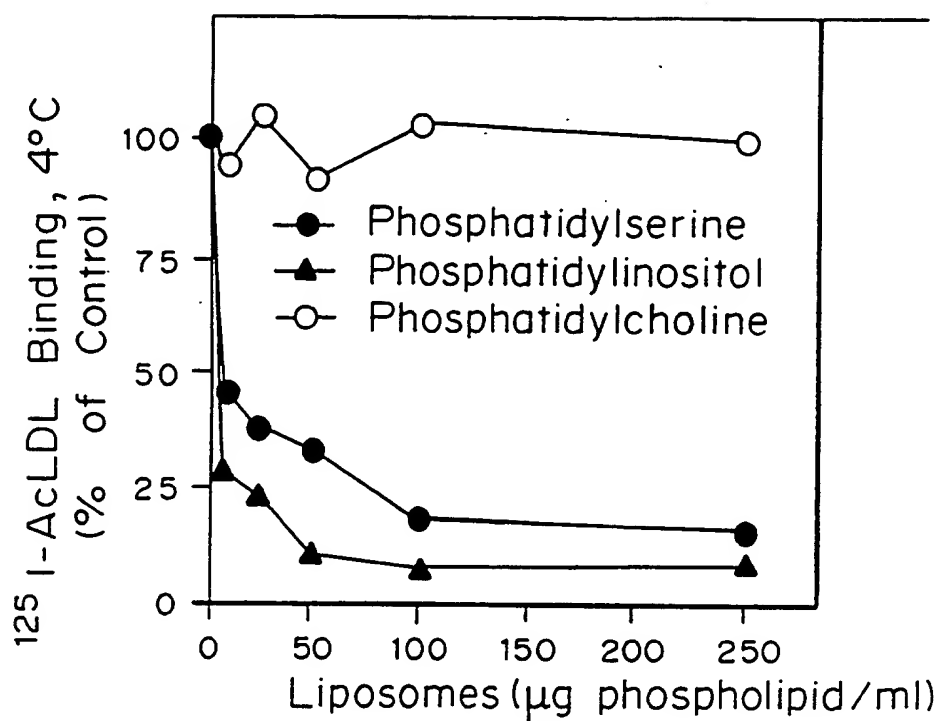


FIG. 7a

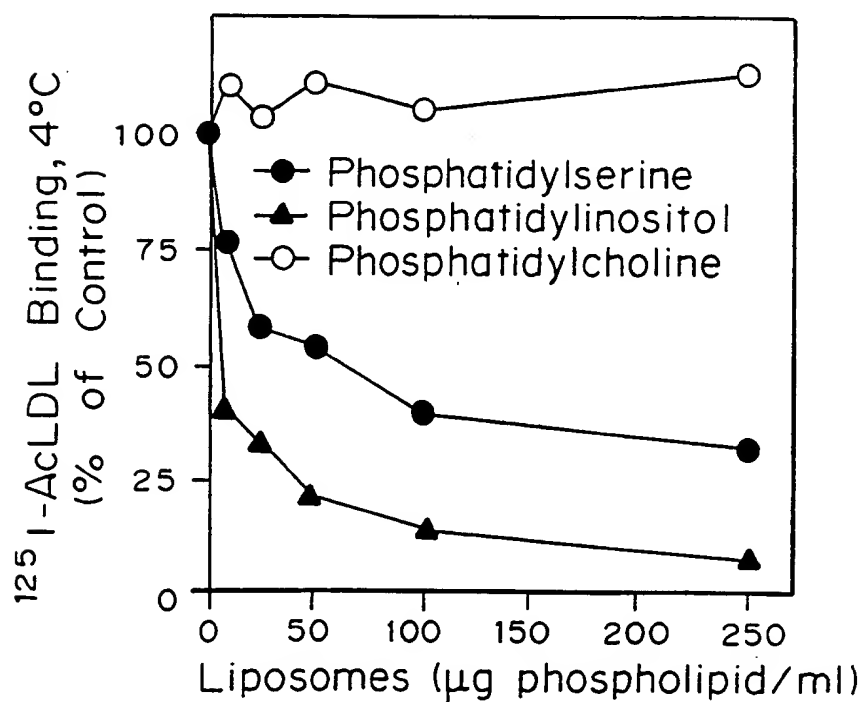


FIG. 7b

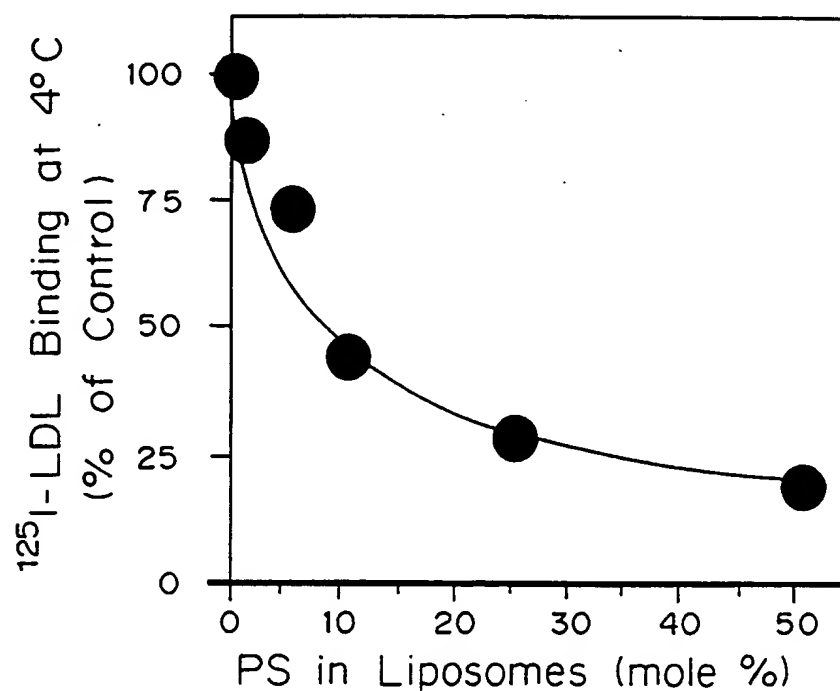


FIG. 7c

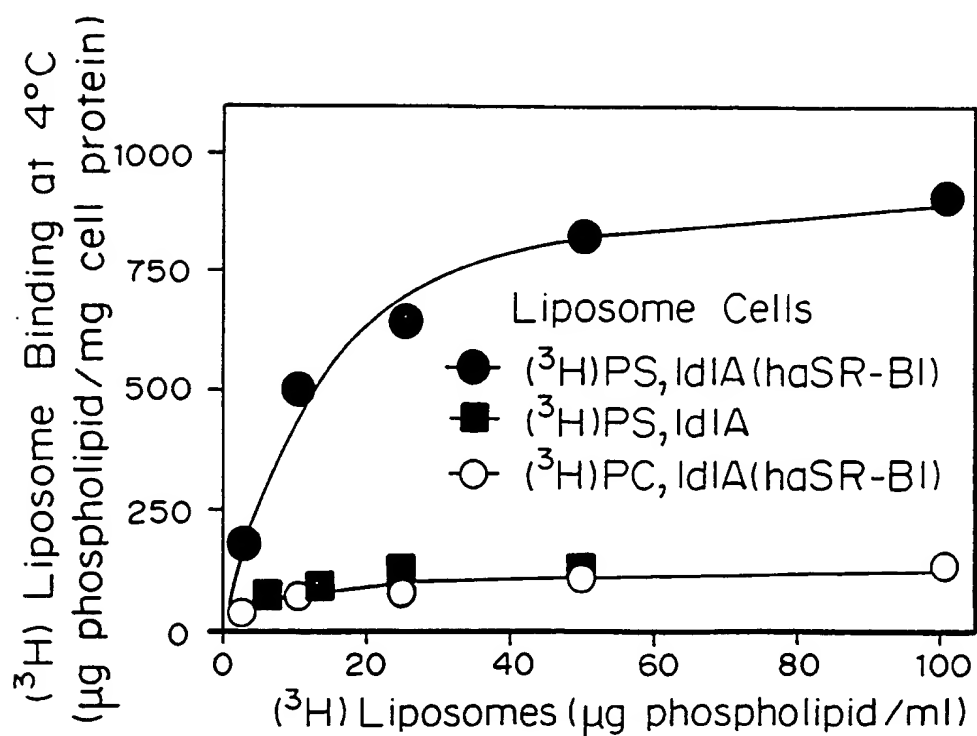


FIG. 7d

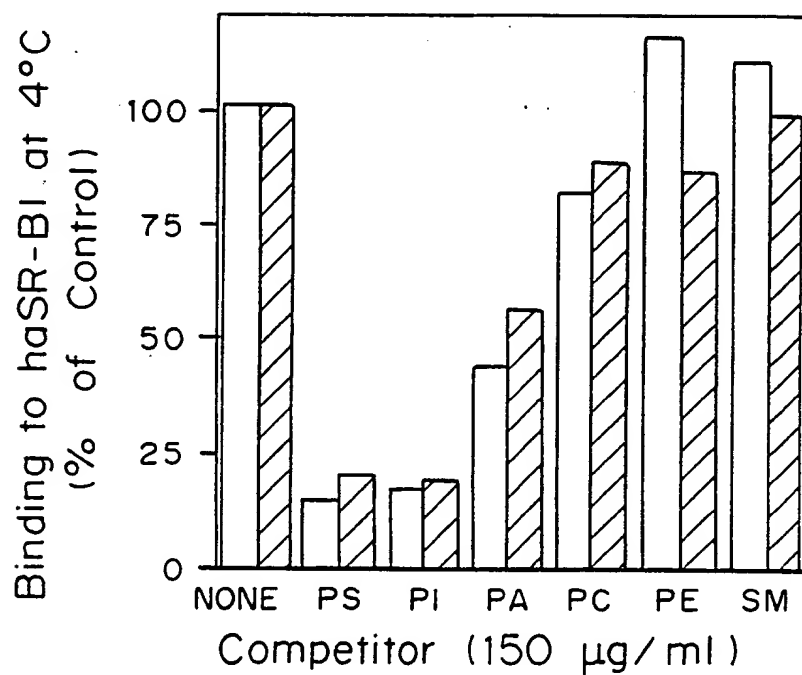


FIG. 7e

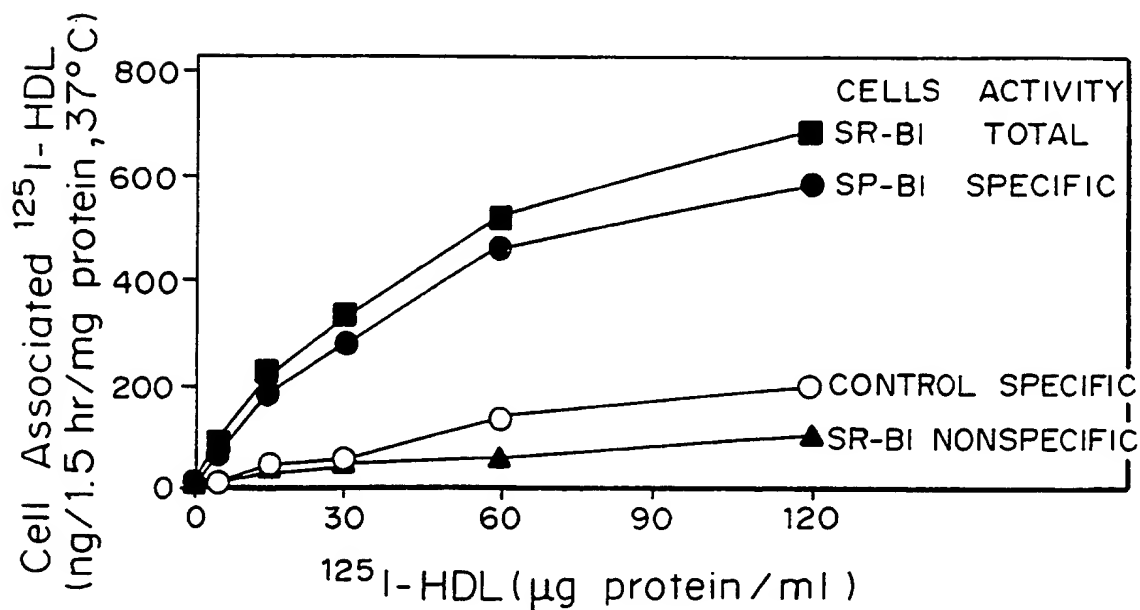


FIG. 8a

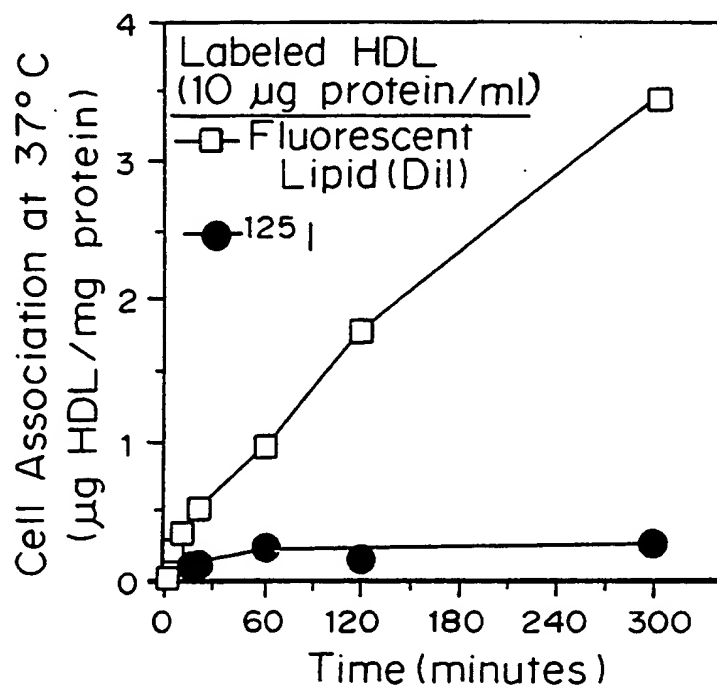


FIG. 8b

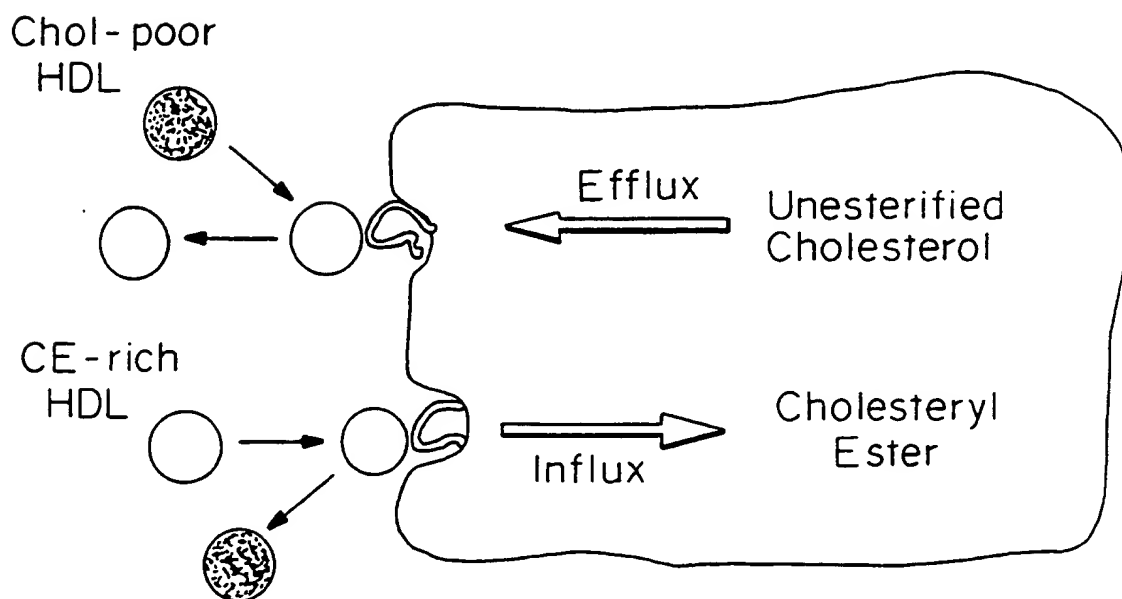


FIG. 9





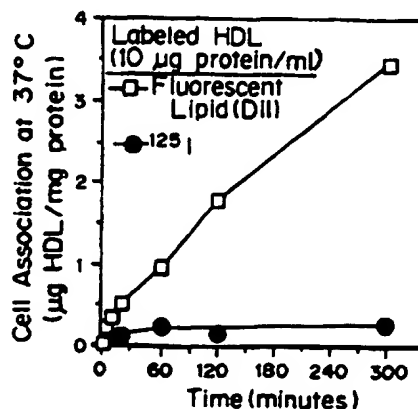
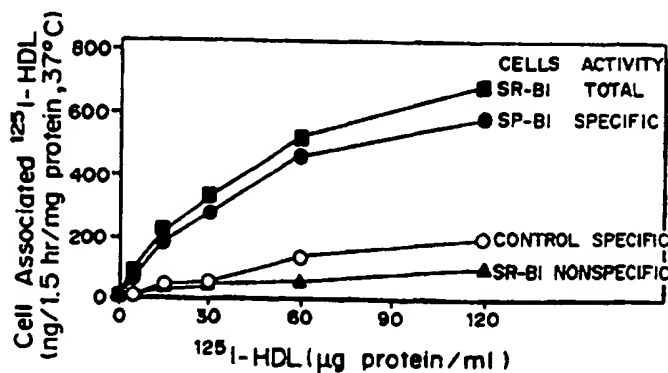
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(51) International Patent Classification <sup>6</sup> : C12N 15/12, C07K 14/705, 16/28, A61K 38/17, C12Q 1/68, G01N 33/53 // C12N 15/11, C07H 21/04		A3	(11) International Publication Number: WO 96/00288
(21) International Application Number: PCT/US95/07721		(43) International Publication Date: 4 January 1996 (04.01.96)	
(22) International Filing Date: 19 June 1995 (19.06.95)		(74) Agent: PABST, Patrea, L.; Amall Golden & Gregory, 2800 One Atlantic Center, 1201 West Peachtree Street, Atlanta, GA 30309-3450 (US).	
(30) Priority Data: 265,428 23 June 1994 (23.06.94) US		(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, FI, GE, HU, IS, JP, KG, KP, KR, KZ, LK, LT, LV, MD, MG, MN, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).	
(60) Parent Application or Grant (63) Related by Continuation US 08/265,428 (CIP) Filed on 23 June 1994 (23.06.94)		Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	
(71) Applicant (for all designated States except US): MASSACHUSETTS INSTITUTE OF TECHNOLOGY [US/US]; 77 Massachusetts Avenue, Cambridge, MA 02139 (US).		(88) Date of publication of the international search report: 4 April 1996 (04.04.1996)	
(72) Inventors; and (75) Inventors/Applicants (for US only): KRIEGER, Monty [US/US]; 139 Woodbine Circle, Needham, MA 02194 (US). ACTON, Susan, L. [US/US]; Apartment 1, 165 Hudson Street, Somerville, MA 02144 (US). PEARSON, Alan, M. [US/US]; 57A Cherry Street, Somerville, MA 02144 (US). RIGOTTI, Atilio [CL/CL]; Apartment 601, 2000 Commonwealth Avenue, Brighton, MA 02135 (US).			

(54) Title: CLASS BI AND CI SCAVENGER RECEPTORS

## (57) Abstract

Two distinct scavenger receptor type proteins having high affinity for modified lipoproteins and other ligands have been isolated, characterized and cloned. HaSR-BI, an AcLDL and LDL binding scavenger receptor, which is distinct from the type I and type II macrophage scavenger receptors, has been isolated and characterized and DNA encoding the receptor cloned from a variant of Chinese Hamster Ovary Cells, designated Var-261. dSR-CI, a non-mammalian AcLDL binding scavenger receptor having high ligand affinity and broad specificity, was isolated from *Drosophila melanogaster*. The isolated receptors are useful in screening for drugs that inhibit uptake of cholesterol in endothelial or adipose cells or macrophages, respectively. They are also useful as probes for the isolation of other lipoprotein receptors and in research the roles of these receptors.



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## INTERNATIONAL SEARCH REPORT

Inv. International Application No.

PCT/US 95/07721

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/705 C07K16/28 A61K38/17 C12Q1/68  
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## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 33, 19 August 1994 page 21003-21009 S. ACTON ET AL. 'Expression Cloning of SR-BI, a CD36-related Class B Scavenger Receptor' see the whole document ---	1-8, 11-22
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY 270 (27). 1995. 16221-16224. ISSN: 0021-9258, RIGOTTI A ET AL 'The class B scavenger receptors SR-BI and CD36 are receptors for anionic phospholipids.' see the whole document ---	1,2,6,7
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Date of the actual completion of the international search

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Date of mailing of the international search report

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P,X	JOURNAL OF IMMUNOLOGY 155 (1). 1995. 367-376. ISSN: 0022-1767, KOBZIK L 'Lung macrophage uptake of unopsonized environmental particulates. Role of scavenger-type receptors.' see the whole document ---	1,2,6,7
X	NATURE, vol. 343, 8 February 1990 pages 531-535, T. KODAMA ET AL. 'Type I macrophage scavenger receptor contains alpha-helical and collagen-like coiled coils' see the whole document ---	1,2,11, 12, 16-18, 20-22
X	J BIOL CHEM 268 (7). 1993. 4569-4572. CODEN: JBCHA3 ISSN: 0021-9258, KRIEGER M ET AL 'MOLECULAR FLYPAPER HOST DEFENSE AND ATHEROSCLEROSIS STRUCTURE BINDING PROPERTIES AND FUNCTIONS OF MACROPHAGE SCAVENGER RECEPTORS.' see the whole document ---	1,7,8, 11,16-19
X	JOURNAL OF CLINICAL INVESTIGATION 93 (5). 1994. 2014-2021. ISSN: 0021-9738, LUOMA J ET AL 'Expression of alpha-2-macroglobulin receptor low density lipoprotein receptor-related protein and scavenger receptor in human atherosclerotic lesions.' see the whole document ---	1,2,7
X	BIOCHEM. J. (1994), 304(1), 69-73 CODEN: BIJOAK;ISSN: 0264-6021, 1994 DE RIJKE, YOLANDA B. ET AL 'Binding characteristics of scavenger receptors on liver endothelial and Kupffer cells for modified low-density lipoproteins' see the whole document ---	1,2,7
X	WO,A,90 05748 (MASSACHUSETTS INST TECHNOLOGY) 31 May 1990 whole document in particular pages 13 and 23 ---	1,2,6,7, 9-12
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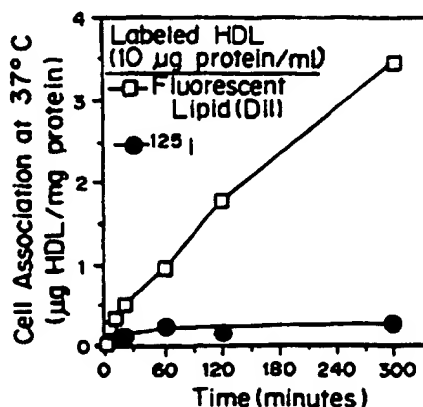
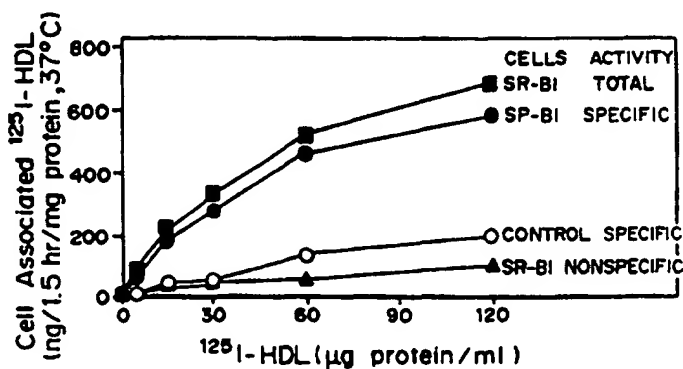
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P,X	JOURNAL OF BIOLOGICAL CHEMISTRY 270 (27). 1995. 16221-16224. ISSN: 0021-9258, RIGOTTI A ET AL 'The class B scavenger receptors SR-BI and CD36 are receptors for anionic phospholipids.' see the whole document ---	1,2,6,7

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